Universidade de Aveiro Departamento de Biologia
Ano 2012

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Hydrobia ulvae: 15 anos de monitorização do *imposex* na Ria de Aveiro

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Biologia Marinha, realizada sob a orientação científica do Doutor Carlos Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Susana Galante-Oliveira, Investigadora em Pós-Doutoramento do Centro de Estudos do Ambiente e do Mar (CESAM).

o júri

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palavras-chave Tributilestanho; TBT; Poluição por TBT; *Imposex*; *Hydrobia ulvae*; Sedimentos; Ria de Aveiro; Portugal

resumo

Compostos de Tributilestanho (TBT) são biocidas com um largo espectro de ação, utilizados em inúmeras aplicações industriais. Nos anos 60, estes compostos foram utilizados como agente ativo em tintas anti-vegetativas de barcos, bóias, plataformas e outras estruturas submersas, para prevenção de bioincustração. Quando libertados nos ecossistemas aquáticos a sua toxicidade provoca diversos efeitos biológicos em espécies não-alvo. Um desses efeitos é o desenvolvimento de caracteres sexuais masculinos sobre o trato reprodutivo de fêmeas de gastrópodes prosobrânquios, um fenómeno apelidado por *imposex*. A expressão do *imposex* é um efeito direto da exposição *in situ* ao TBT e é utilizado como biomarcador da sua poluição. As várias e generalizadas descrições de elevados níveis de poluição por TBT levaram à implementação de legislações extremamente restritivas da utilização destes compostos. Atualmente, e desde 2008, estão interditas a aplicação e circulação de compostos de TBT em embarcações e portos de estados membros da União Europeia, bem como em embarcações de países que assinaram a Convenção Internacional para a Convenção de Sistemas Antivegetativos Prejudiciais em Navios (Convençao AFS). Contudo, e apesar de estar reportado uma redução na poluição ambiental por TBT após estas medidas, estes compostos podem estar a ser utilizados em países em desenvolvimento, o que requer uma monitorização constante. Além disso, mesmo onde os *inputs* não são permitidos, a monitorização de sedimentos é requerida, pois este compartimento constitui um reservatório de TBT, acumulado durante as décadas de uso intenso. Várias espécies bioindicadoras podem ser utilizadas para avaliação do *imposex* e monitorizar a poluição por TBT. Estas devem ser selecionadas cuidadosamente, dependendo da sua abundancia e distribuição na área de estudo e também do compartimento que está a ser monitorizado. De forma a avaliar o estado da poluição por TBT e a sua evolução temporal durante a última década na Ria de Aveiro (Noroeste de Portugal), em especial nos sedimentos, foi utilizado o gastrópode *Hydrobia ulvae* como espécie bioindicadora. Os 10 locais de amostragem selecionados foram monitorizados em 1998, e em 2003/2004/2007, sendo revisitados em 2012. Os níveis de *imposex* foram avaliados e os compostos organoestânicos (OTs) foram

resumo (cont.)

quantificados nos sedimentos. Ocorreu uma diminuição geral das concentrações de TBT em sedimentos entre 1998 e 2012. No entanto, os níveis de *imposex* na *H. ulvae* não diminuíram durante o mesmo período. Em vez disso, houve um aumento global da percentagem de fêmeas afetadas pelo *imposex* (variou entre 76-100%) e uma manutenção geral do índice da sequência do vaso deferente (VDSI; oscilou entre 0,93 e 1,17). Assim, existe outra razão para além da exposição ao TBT que causa a manutenção da intensidade do *imposex* e a prevalência deste fenômeno, que aumenta ao longo deste sistema estuarino. Na verdade, a percentagem de indíviduos parasitadas também aumentou ao longo da área de estudo entre 2003 e 2012. Aliás, todas as fêmeas infestadas exibiam *imposex* e nenhuma fêmea não afetada pelo *imposex* estava parasitada.

Assim, o fenómeno do *imposex* pode ser uma consequência do aumento deste parasitismo em populações *Hydrobia ulvae* na Ria de Aveiro, facto corroborado pelos d

ados recolhidos no estudo presente. Através de análises estatísticas foi demostrado um efeito positivo da presença de parasitas nos índices VDSI ($P = 1{,}4 \times 10^{-3}$) e FPL (tamanho do pénis feminino; $P = 8{,}4 \times 10^{-5}$), ao contrário do efeito das concentrações de TBT nos sedimentos nos mesmos parâmetros ($P = 0{,}62$ e $P = 0{,}99$, respectivamente). No entanto, mais estudos são necessários para confirmar esta hipótese de que pode restringir o uso desta espécie como bioindicador da poluição por TBT em populações parasitadas.

keywords

Tributyltin; TBT; TBT pollution; *Imposex*; *Hydrobia ulvae*; Sediments; Ria de Aveiro; Portugal

abstract

Tributyltin (TBT) compounds are broad spectrum biocides with multiple industrial applications. They have been used as active ingredients in anti-fouling paints formulations, at a global scale since the 60s, and applied in vessels hulls, buoys, platforms and other submerged structures to prevent bioincrustation. Once released into the aquatic environment, and given its proven toxicity, several adverse biological effects are induced on non-target species. One of these effects is the development of male sexual characters onto the reproductive track of prosobranch gastropods females, a phenomenon termed *imposex*. *Imposex* expression is a direct effect of TBT exposure *in situ*, and has been widely used as a biomarker for TBT pollution. The numerous and generalized descriptions of high levels of TBT pollution and also its the negative effects led to the implementation of extremely restrictive legislation on these compounds usage. Presently, and since 2008, TBT compounds application and circulation are banned from EU member states fleets, and respective ports, and from ships flying flags of countries signatories of the International Convention for the Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention). However, and despite reports of TBT environmental pollution reduction after such measures, these compounds can still be used in third countries, requiring continued monitoring. Furthermore, even where inputs are now prohibited, sediment monitoring is required since this compartment constitutes a reservoir of TBT accumulated during decades of intense use. Several bioindicator species can be used for *imposex* assessment and TBT pollution monitoring. Therefore, the bioindicator should be carefully selected depending on the abundance and distribution in the study area, and also on the compartment being monitored. In order to evaluate the TBT pollution current status and its temporal evolution in the last fourteen years in Ria de Aveiro (NW Portugal), namely its persistence in sediments, the gastropod *Hydrobia ulvae* was selected as a bioindicator. The 10 sampling sites previously surveyed in 1998 and in 2003/2004/2007 were revisited in 2012. *Imposex* levels were assessed and the organotins (OTs) quantified in sediments. There was a general decrease of TBT concentrations in sediments between 1998 and 2012. Even so, *H. ulvae imposex* levels did not decrease in the same period. Instead, there was a global increase in the percentage of females affected by *imposex* (varied between 76 and 100%) and a general maintenance of the vas

abstract (cont.)

deferens sequence index (VDSI; from 0.93 to 1.17). It is therefore obvious that other reason than TBT exposure is causing *imposex* intensity maintenance and the phenomenon increased prevalence through this estuarine system. Actually, the percentage of parasitized specimens also increased throughout the study area between 2003 and 2012. Moreover, all infected females exhibited *imposex* and none *imposex*-affected female was parasitized.

Thus, the *imposex* phenomenon might be a consequence of this increased parasitism in populations of *Hydrobia ulvae* in the Ria de Aveiro, a fact corroborated by the data collected in this study. Through statistical analyzes, it was demonstrated a positive effect of the presence of parasitic organisms in VDSI ($P = 1.4x10^{-3}$) and FPL (female penis length; $P = 8.4x10^{-5}$), contrasting with the effect of TBT concentration in sediments, in the same *imposex* parameters ($P = 0.62$ and $P = 0.99$, respectively). However, further studies are needed to confirm this hypothesis that can constrain the use of this species as a bioindicator of TBT pollution in parasitized populations.

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1. Tributyltin (TBT) Pollution

1.1. Chemistry of TBT

The first organotin compound (OT), diethyltin-diiodide, was synthesized in 1853 by Sir Edward Frankland (Hoch, 2001). Since then, more than 800 tributyltin (TBT) compounds were manufactured, although their economically viable function was only found in the 40's (Hoch, 2001). Organotins (OTs) were initially used in the plastic industry as PVC stabilizers (Han et al., 2009; Hoch, 2001; Takahashi et al., 1999a). Thereafter, numerous functions arose such as their application as industrial catalysts, polyurethane foams, silicones, agricultural pesticides and antifouling (AF) agents – i.e. as biocides in AF paints formulations applied in submerged structures namely in vessels and aquaculture equipment to prevent the bioincrustation phenomenon (Omae, 2003).

Amongst OTs, TBT and triphenyltin (TPT) compounds were the most widely used as biocides. Tributyltin (Fig. 1) and TPT are chemical moieties in which the metal ion Tin (Sn) is covalently bonded to three *n*-butyl and *n*-phenyl chains, respectively (Hoch, 2001; WHO, 1990).

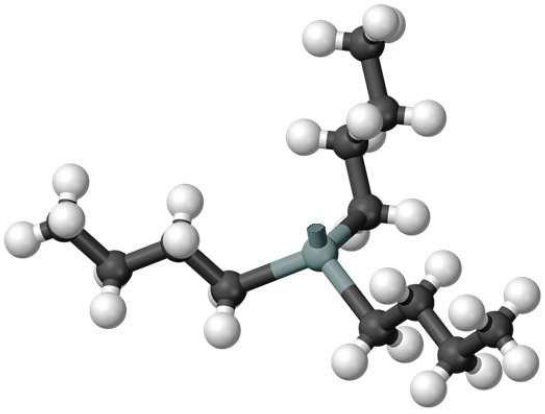

Figure 1 – TBT moiety. In gray is shown the tin ion bonded to three n-butyl groups. Adapted from Galante-Oliveira, 2010.

Tributyltin compounds enter aquatic ecosystems through leaching and/or scraping surfaces coated with TBT-based AF paints (e.g. vessels hulls; Bech, 2002). Once in the water compartment, TBT compounds are degraded in less toxic compounds by the sequential loss of butyl groups originating dibutyltin (DBT), monobutyltin (MBT) and finally inorganic tin (see Fig. 2; Hoch, 2001; Pope, 1998).

This process is dependent on both biotic and abiotic factors, such as biological and chemical cleavage, OTs environmental concentration, temperature, salinity, UV radiation and light exposure (Batley, 1996; de Carvalho Oliveira & Santelli, 2010; Hoch, 2001; Omae, 2003; Pope, 1998). The greater these factors are, and the lower TBT concentration is, more accelerated is the TBT degradation (Batley, 1996; Sakultantimetha et al., 2011). Accordingly, and because those factors intensity are different in different ecosystem compartments, TBT half-life in the water column is much lower than the predicted for the sediment: 6 to 7 days at 28°C in water (and in sediments surface layer; de Mora, 1996; Sakultantimetha et al., 2011); while in deeper anoxic layers the degrading process is much slower, with reported half-lives between 1.9 and 3.8 years (Batley, 1996; Sakultantimetha et al., 2011). This extended degradation in sediments, together with the wide-ranging and constant input by the TBT-based AF paints circulation during decades, implied that sediments have been acting as reservoirs, carrying on the compound slowly release (Langston & Pope, 1995; Burton et al., 2006; Ruiz et al., 2008).

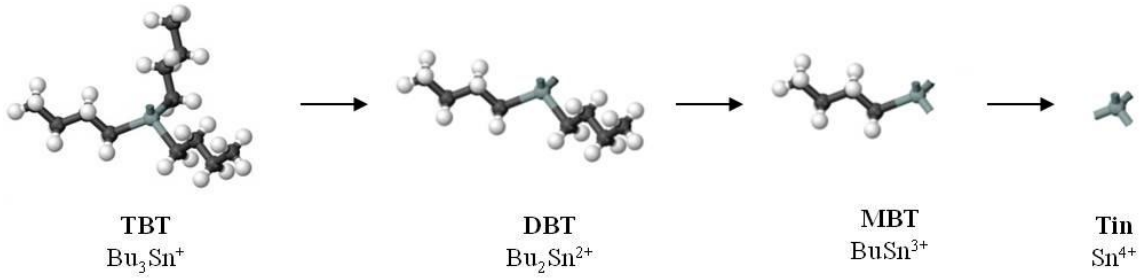

Figure 2 – TBT degradation scheme. Sequential loss of *n*-butyl chains (Bu) originating dibutyltin (DBT), monobutyltin (MBT) and inorganic tin (Sn). Adapted from Galante-Oliveira, 2010.

TBT-based antifouling (AF) paints

Since ancient times, biofouling is a recognised and combated concern for mariners (Callow & Callow, 2002; Yebra et al., 2004). Since ancients' cultures, antifouling methods were applied in ships' hulls, e.g. Phoenicians and Greeks were said to have used copper sheathing – although its antifouling characteristics were only studied and confirmed in the 19th century; while the Romans favoured lead sheathing (Callow & Callow, 2000; Yebra et al., 2004).

Antifouling (AF) paints were developed in the mid-1800s and contained organometallic compounds, such as cuprous oxide (Cu_2O), arsenic (As) and mercury oxide (HgO; Yebra et al., 2004). The replacement of Cu_2O by the use of TBT compounds as active ingredients in AF paints occurred in the mid-1960s (Barroso et al., 2000; Titley-O'Neal et al., 2011) and in the 70s most of the world fleet wore TBT based AF paints. These systems were very successful due to: (i) the high efficiency and durability; (ii) the compounds colourless, which allowed the application of any colour chosen by the vessel owner, without losing the biocidal properties; (iii) the idea that TBT compounds would be environmentally safe biocides, less toxic than the ones used hitherto for the same purpose (Galante-Oliveira, 2010; Pope, 1998; Silva, 2002). However, these paints produced an extremely toxic micro-film to aquatic fouling organisms, due to the biocide leaching from the paint in high concentrations (Hoch, 2001). This biocide release occurred by two pathways, depending on the AF system used: (i) in free-association AF paints, TBT compounds were embedded into a matrix, that could be insoluble (contact leaching paints) or soluble (ablative paints), being released once in contact with the water (high leaching rate and loss of effect after 18 to 24 months); (ii) in self-polishing AF paints the biocide was chemically bounded to a polymer and gradually released from the painting surface to control the leaching rate (constant and relatively slow release of TBT, increasing the painting life to a maximum of 60 months; de Mora, 1996; Hoch, 2001).

Soon after the TBT release, adverse effects in some non-target species such as bivalves, gastropods, crustaceans and fish, were observed (Bettin et al., 1996; Galante-Oliveira et al., 2011; Oehlmann & Schulte-Oehlmann, 2003; Titley-O'Neal et al., 2011).

Toxicity of TBT

The excessive use of TBT-based AF paints resulted in a worldwide problem of TBT bioaccumulation with toxic biological effects in non-target species (Takahashi et al., 1999a).

The first indication of TBT toxicity was reported at the Arcachon Bay – France (Alzieu, 2000; Galante-Oliveira, 2010; Oehlmann & Schulte-Oehlmann, 2003; Pope, 1998), an area that encloses several marinas and fishing ports and where several thousand tons of oysters are produced annually for marketing and worldwide distribution (Alzieu, 2000). At the end of the 70s, oysters' production was severely affected by sudden reductions in the specimens' growth rates, absence of reproduction and abnormalities on the calcification process (Alzieu, 2000). After several studies and field observations, these anomalies were related to the TBT release into water at numerous marinas located in the bay (Alzieu, 2000). Some years later, equal effects on oysters were recorded in Australia and New Zealand (de Mora, 1996). These physiological responses were dependent on the TBT environmental concentration (Alzieu, 2000).

TBT compounds are responsible for changes in reproductive cycles, inhibit sexual maturity, larvae growth and impose overlapping sex on bivalves (Alzieu, 2000; Barqueiro-Cárdenas et al., 2007). It is also harmful for gastropods, inducing females' masculinization and decreasing the breeding potential (Barqueiro-Cárdenas et al., 2007). This phenomenon is termed *imposex* (see in section 1.2.) and occurs for TBT concentrations under 1 ng TBT-Sn.l^{-1} in water (Bryan et al., 1987).

Due to its characteristics and harmful effects to non-target organisms, TBT compounds were considered one of the most toxic xenobiotics produced by mankind and released deliberately in to ecosystems (Goldberg, 1986).

Regulating the use of TBT

The harmful effects of TBT compounds in oysters farming prompted the first legal restriction on the use of TBT-based AF paints, in 1982 by the French government. The measure prohibited the use of those paints on vessels <25 meters in length, in areas of intense oysters' production and later on the entire French coastline (Galante-Oliveira, 2010; Stewart, 1996).

The TBT ban in France urged the same measures in industrialized countries as United Kingdom in 1987; United States and New Zealand in 1988; Canada, Australia, Norway and Japan in 1989/90 (IMO, 2002). Afterwards, it was observed a certain TBT pollution decrease and amelioration of oysters' calcification and gastropods' *imposex* levels in some locations (Batley, 1996). Even so, unwanted effects on non-target organisms continued to be registered in places with high traffic of large vessels. As a consequence, in 1988, the Marine Environmental Protection Committee (MEPC) of the International Marine Organization (IMO) considered the need for measures under relevant legal instruments to restrict the use of TBT compounds on sea-going vessels (IMO, 2002) and, in the same year, the European Commission implemented a legal restriction on the TBT use – the Council Directive 89/677/ECC (Ref. No. OJ L, 1989). Since then, the use of TBT compounds was banned in AF paints in hulls of vessels less than 25 metres in length, in equipment used for fish or shellfish farming, in any totally or partly submerged equipment and in industrial water treatment. The directive was transposed into the Portuguese National Law in 1993, although showed to be ineffective in reducing the biological effects of the TBT pollution (Barroso et al., 2002; Barroso et al., 2000; Galante-Oliveira et al., 2011; Santos et al., 2002), scenario also observed in other EU Member States particularly where the traffic of larger ships is more expressive.

In an attempt to globally eradicate this problem, the MEPC adopted the "International Convention on the Control of Harmful Antifouling Systems on Ships" (AFS Convention) on 5 October 2001, seeking the global ban on TBT compounds usage. Once ratified, this document foresaw the prohibition to apply and reapply TBT-based AF paints on ships hulls from 1 January 2003, and the interdiction on its use and circulation by 1 January 2008. However, the AFS Convention was opened for signature on the 1st February 2002 and would only be effective 12 months after being ratified by 25 countries, whose fleets represent not less than 25% of the world's merchant shipping tonnage (IMO, 2001). Therefore, it would be extremely difficult to accomplish the dates instituted in the AFS Convention. Nevertheless, those actions were imposed to all EU Member States maritime area on the AFS Convention scheduled dates through the mandatory implementation of the EC Regulation

782/2003. Thus, it was legally instituted the global interdiction on the application of TBT-based AF paints on ships flying the flag of, or operating under the authority of, a EU Member State, and on ships, regardless of the flag they fly, sailing to or from ports of the Member States, by the 1st July 2003, and the total ban since the 1st January 2008.

The AFS Convention entry into force was finally met on 17 September 2007, once 38% of the world's merchant shipping tonnage had ratified it, in representation of 25 countries. However, only 12 months later it could be legally entered into force. Therefore, the use of TBT compounds as biocides in AF systems was globally forbidden since 17 September 2008 (IMO, 2009). The efficacy of all these restrictive measures has been evaluated over time by both chemical and biological TBT pollution monitoring.

1.2. TBT pollution monitoring

The TBT pollution in aquatic environments can be assessed by both chemical and biological monitoring (MEPC, 2008).

As mentioned before, TBT compounds degradation rate is faster in the water column and sediments surface layer (oxygenated) than in sediments deep anoxic layers mainly due to the high adsorption of TBT to sediment particles (de Mora, 1996; Maguire, 1996). This fact, together with the wide-ranging and constant input by the TBT-based AF paints circulation during decades, sediments have been acting as major long-term reservoirs of TBT, carrying these compounds slowly release (Langston & Pope, 1995; Ruiz et al., 2008). Therefore, much more than water, the chemical monitoring of sediments is indicative of the TBT persistence in the ecosystem and of possible fresh inputs in the aquatic environment even after their prohibition (de Mora, 1996; Langston & Pope, 1995; Sousa et al., 2009a).

In addition to OTs chemical analysis in water and sediment, monitoring the environmental pollution of these compounds can also involve their quantification in

organisms' tissues – Biota chemical analysis. Nevertheless, this compartment can also be monitored otherwise than chemically by the assessment of the biological effects of the pollutants (Galante-Oliveira, 2010; Rainbow, 2006).

The International Mussel Watch Program is a global-scale monitoring program and has been in existence since the mid-1970, along the South America, Central America, Caribbean and Mexico coasts. This program concept is based on the analysis of bivalves' tissues – from mussels and oysters – in order to assess the spatial distributions and the temporal trends of a suite of organic contaminants and trace metals in coastal and estuarine regions. The selected monitoring methodology was successfully applied in other programs, either national or regional, in Europe, Taiwan, Canada and in the United States (International Mussel Watch Committee, 1995).

Biomonitoring (or monitoring of Biota) has been intensively developed, not only regarding the optimization of methods for the detection of chemicals in organisms (chemical monitoring of Biota) but also defining new parameters and establishing new methods to assess the biological effects of many contaminants, namely by the identification and validation of "biomarkers" that can be used to evaluate the exposure to / the effect of / the individual susceptibility to a chemical (National Research Council, 1987).

In the Northeast Atlantic, the Oslo and Paris (OSPAR) Commission is the entity responsible for the protection of the marine environment and adopted specific guidelines to monitor TBT pollution in the area under its jurisdiction, including the maritime area of Portugal (see OSPAR Agreement 2004-15). As recommended by OSPAR Joint Assessment and Monitoring Program (JAMP) Guidelines for Contaminant-Specific Biological Effects, the biomarkers for TBT pollution biomonitoring are *intersex* and/or *imposex* (see the next section), (MEPC, 2008).

Imposex – the TBT exposure biomarker

Imposex – or pseusohermaphrodism – is the superimposition of male sexual characteristics – growth of a penis and/or a vas deferens and prostate tissue – onto prosobranch females' reproductive tract (Smith, 1971 *in* 1981). It alters the organisms' physiology, provokes reproductive abnormalities and can cause female sterilization (Smith, 1981). The first description of *imposex* occurred in 1970 by Blaber, but it was termed by Smith in 1971 and its correlation with the environmental exposure to TBT was unveiled only 10 years later by the same author (Smith, 1981). This phenomenon is one of the best documented examples of endocrine disruption ever described and had been used as a biomarker of the TBT environmental concentrations in many studies, during the last decades (Matthiessen & Gibbs, 1998).

In the late 80's, by exposing females of the prosobranch *Nucella lapillus* to increasing concentrations of TBT, Gibbs and Bryan (1986) concluded that *imposex* is initialized at 1-2 ng TBT-Sn.l^{-1} (i.e. concentrations in water below its chemical detention; < 0.5 ng TBT-Sn l^{-1}) and that at 5 ng TBT-Sn.l^{-1} females got sterilized due to the oviduct blockage (Gibbs & Bryan, 1996; Huet et al., 1996). The worldwide use of TBT-base AF paints and the continuous release of TBT compounds during decades led to extremely high levels of pollution by these compounds in areas of intense naval traffic, nearby marinas, sheltered estuaries and navigation channels, where prosobranch gastropods populations suffered drastic abundance reductions or even extinction[1] (Bettin et al., 1996; Gibbs & Bryan, 1996; Huet et al., 1996; Morgan et al., 1998).

Since *imposex* first description (1970), it was described for more than 240 prosobranch species and several have been used as TBT pollution bioindicators (Titley-O'Neal et al., 2011). Throughout the analysis of the TBT compounds biological effects on these bioindicators, studies concluded that:

[1] *Nucella lapillus*'s population from the inner shore of Plymouth Sound (England) were recorded in late-1980s and was composed by old individuals, mainly males and sterile females (Gibbs & Brian, 1996; Huet et al., 1996).

(i) TBT bioaccumulation can be direct – by the contact of the organism with contaminated water and/or sediments – or indirect – through contaminated food ingestion (Huet et al., 1996; Pope, 1998; Silva, 2002);

(ii) *Imposex* intensity is dose-dependent and species-specific (Huet et al., 1996; Schulte-Oehlmann et al., 1997; Oehlmann & Schulte-Oehlmann, 2003; Schulte-Oehlmann et al., 1998; Silva, 2002);

(iii) The phenomenon is induced at very low TBT concentrations (Gibbs & Bryan, 1986; Huet et al., 1996; Schulte-Oehlmann et al., 1998);

(iv) *Imposex* is an irreversible process, so organisms can express a contamination level which no longer corresponds to the TBT concentration *in situ* (Galante-Oliveira, 2010; Silva, 2002);

(v) *Imposex* consequences are species-specific (Silva, 2002).

The *imposex* expression intensity is related to the TBT exposure concentration, i.e., by the phenomenon quantification it is possible to estimate the regime to which the animal was exposed (Gibbs, 1999). This quantification is of simple observation and determination using several indices developed over time with this purpose.

Indices

Several indices were developed to characterize and quantify *imposex* expression incidence and severity (see the extensive review on OTs effects on gastropod females by Horiguchi, 2012 and Titley-O'Neal et al., 2011). More than 20 different indices have been used during more than half a century of *imposex* studies (Titley-O'Neal et al., 2011), yet the classic and most frequently used (namely in the present work) are as follows.

(i) The Vas Deference Sequence Index (VDSI) measures the *imposex* intensity in a population. It corresponds to the arithmetic mean of the Vas Deferens Sequence (VDS) stage assigned to each individual by comparison with scoring systems based on the vas deferens development in that species. In *H. ulvae* the VDS classification

scheme comprises 5 stages, from 0 – "unaffected female" to 4 – "vas deferens completely developed" (see section 2.4.), (Schulte-Oehlmann et al., 1997).

(ii) The penis is another male sexual organ imposed on *imposex*-affected females and so its measurement is also indicative of *imposex* intensity/severity. Thus the mean Penis Length (PL), expressed in millimetres (mm), is determined for both females and males in the same population, resulting in two distinct indices: mean Female Penis Length (FPL) and mean Male Penis Length (MPL).

(iii) The Relative Penis Length Index (RPLI) is calculated by $\frac{FPL}{MPL} \times 100$ and expresses the mean length of the females' penises as a percentage of that of the males of the same population (Schulte-Oehlmann et al., 1997) allowing FPL comparisons between different populations. It is an adjustment of the initially developed Relative Penis Size Index (RPSI), introduced by Gibbs et al. in 1987 to express *Nucella lapillus* females penis volume / size (and not only the length) as a percentage of that of the males for a given population. This later index is calculated by $\frac{FPL^3}{MPL^3} \times 100$ since this muricid penis is a voluminous organ. Moreover, *imposex* in *N. lapillus* was always performed without narcotization, contrarily to other species namely *H. ulvae*. In fact, the narcotization is proved to induce the tissue distension, an effect more pronounced in males, so increasing MPL and resulting in RPSI underestimation (Gibbs, 1999; Galante-Oliveira et al. 2010a). Accordingly, the RPLI is subjected to less variation thus indicated for species which specimens are observed after a narcotization period, or that have been cryopreserved (Gibbs, 1999).

(iv) The Percentage of females affected by *Imposex* (**%I**) is a measure of the prevalence of the phenomenon in the sampled population indicating, within the universe of the analysed females, which percentage exhibit the phenomenon. However, it shows to be ineffective to distinct the pollution severity from different sites. It was applied from *imposex* first descriptions (e.g. Gibbs et al., 1987) and is used for most species including *Hydrobia ulvae* (Schulte-Oehlmann et al., 1997).

(v) The percentage of Sterile females (%S) measures females sterility in the sampled population indicating, within the universe of the analysed females, which

percentage are sterilized by *imposex* extreme development. It was used to evaluate the impairment of females' reproductive capacity and the possibility of extinction mainly in the most sensitive species such as *Nucella lapillus* (e.g. Gibbs et al., 1987).

(vi) The Average Oviduct Stage (AOS), proposed by Barreiro and co-workers (2001) to assess the degree of oviduct convolution in *Nassarius reticulatus* (Laranjeiro, 2009).

(vii) The Intersex Index (ISI) is a biological parameter and scores the development of intersex in *Littorina littorea* by a scoring specific system (Barroso et al., 2000).

2. *Hydrobia ulvae*

Taxonomic classification

Kingdom **Animalia**

Phylum **Mollusca**

Class **Gastropoda** (Cuvier, 1795)

Subclass **Prosobranchia** (Milne-Edwards, 1848) / **Caenogastropoda** (Cox, 1960)

Order **Hypsogastropoda**

Suborder **Littorinimorpha** (Golikov and Starobogatov, 1975)

Superfamily **Rissooidea** (Gray, 1847)

Familia **Hydrobiidae** (Simpson, 1865)

Genus ***Hydrobia*** (Hartmann, 1921)

2.1. Species biology

Hydrobia ulvae is a small gastropod – shell height of up to 8 mm– with a lifespan of up to 4 years (Gorbushin et al., 2001). This species distributes from Norway to Senegal, the North Sea, the English Channel and the Mediterranean Sea, in waters with a salinity range between 5 and 40 (Anders et al., 2008; Galante-Oliveira, 2010; Silva, 2002). It inhabits estuarine intertidal areas on muddy sediments and salty marshes, selecting fine and/or highly chlorophyll *a* content sediments (Anders et al., 2008; Chandrasekara & Frid, 1998; Galante-Oliveira, 2010; Hampel et al., 2009; Orvain & Sauriau, 2002; Silva, 2002).

As the most abundant species of the intertidal, reaching 500,000 individuals per m^2 (Anders et al., 2008), the mud snail is a major transporter of organic matter between trophic levels and constitutes an important component of estuarine taxa diet, as gastropods, crustaceans, fish and birds (it is, for example, the main

nourishment of the shelduck *Tadorna tadorna*; Anders et al., 2008; Cardoso et al., 2002; Coelho, 2010; Hampel et al., 2009; Mauffret et al., 2010; Silva, 2002).

Hydrobia ulvae's morphology is typical of a gastropod (Fig. 3). Its head contains a dorsal slender proboscis which expands in the terminal region, forming two lobes – in the centre is situated the mouth of the animal – and two eyes which are located at the base of the long cephalic tentacles (Gorbushin et al., 2001). The mud snail is a dioecious species: the female reproductive system is constituted by a gonodial and a renal section – the gonad gland is connected with the capsule glandule through the genital duct (Brusca & Brusca, 2002; Oehlmann-Schulte et al., 1997; Silva, 2002); the male reproductive system is composed by an organ assembly, formed by testis, vas deferens – which extends along the entire length of the system –, prostate gland and penis. The testis occupies a position similar to the female gonad (Brusca & Brusca, 2002; Schulte-Oehlmann et al., 1997).

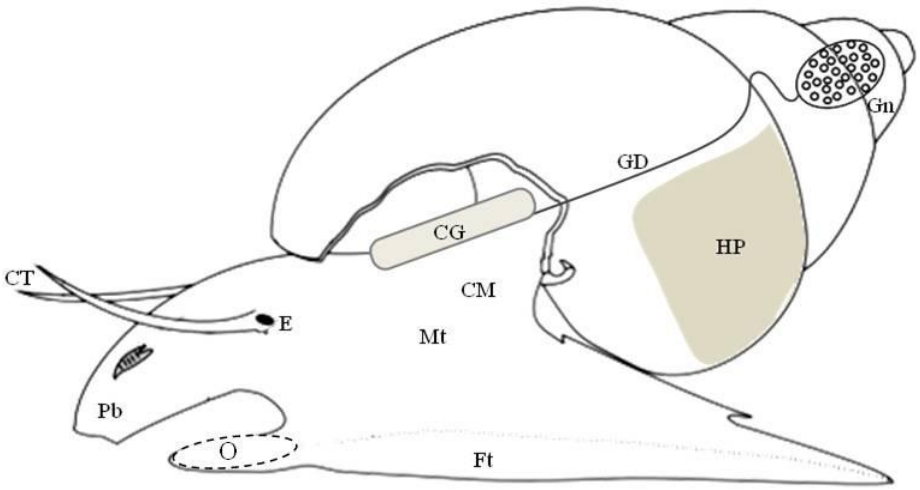

Figure 3 – *Hydrobia ulvae*. Female morpho-anatomical scheme: CT – Cephalic Tentacle; Pb – Proboscis; E – Eye; CM – Columellar muscle; Mt – Mantle; Ft – Foot; O – Operculum; CG – Capsule Gland; GD – Gonoduct; Gn – Gonad; HP – Hepatopancreas. Adapted from Gorbushin et al. (2001).

H. ulvae's diet is based in seagrass as *Ulva sp*. and *Enteromorpha sp*. and in benthic organisms such as bacteria and diatoms. Since its food items availability depends on the tidal cycle, the mud snail displays a daily pattern of vertical migration (Fig. 4), described by Newell (1962 *in* Galante-Oliveira, 2010): during the receding tide, the animals sink and crawl on the sediment full of microorganisms, scraping and feeding. As *H. ulvae* has a burying capacity, specimens bury themselves in the sediment surface layers during low tide to feed on sub-surface material (animals can reach up to 30 mm depth searching for additional food, but 90% of the population stay on the sediment surface or up to 15 mm deep; Chandrasekara & Frid, 1998; Galante-Oliveira, 2010; Orvain & Sauriau, 2002; Silva, 2002). When the tide refills, organisms emerge from the substrate. In these phase, *H. ulvae* produces a mucous raft which gives buoyant capacity, allows dispersion and also facilitates food capture (Orvain & Sauriau, 2002; Silva, 2002; Sola, 1996).

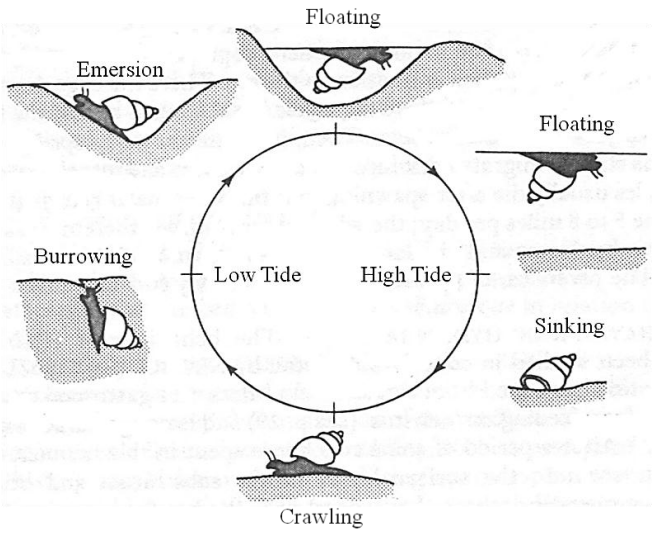

Figure 4 – *Hydrobia ulvae*. Schematic representation of the vertical migration in the water column during one tide cycle, proposed by Newell (1962). Adapted from Galante-Oliveira, 2010.

The floating behaviour of juvenile *H. ulvae* – shell height less than 2 mm – is a very important mechanism: as they are passively transported through the tides and currents, they explore new food sources and allow the species dispersion (Armonies, 1992; Armonies & Hartke 1995; Silva, 2002). This seasonal mobilization changes the populations' age structure and may be crucial for the population density balance at each location, reducing the competition either for space or food (Armonies & Hartke, 1995). Floating aggregates can be composed up to 98% of juvenile individuals (Armonies, 1992); although Meireles (1998) recorded between 77 and 95% of juveniles in a floating aggregate in Ria de Aveiro.

2.2. Reproductive cycle

Hydrobia ulvae is a dioecious species and has an internal fertilization (Silva, 2002; Sola, 1996). As a possible adaptive strategy, its reproductive cycle varies with the population geographical location such as its sexual maturation which can occur between their first and second year of life, while spawning can occur throughout the year or only once a year (Silva, 2002; Sola, 1996).

H. ulvae's eggs are small - from 70 to 90 μm - and released in large gelatinous masses of the ovigerous capsules, which are deposited either on biogenic material (i.e. the shells of live individuals of the same species or empty shells of the same or other species, and algae), on biological material (i.e. algae) or even on sand grains (Silva, 2002; Sola, 1996). Eggs development culminates in a veliger larva hatching, which nature may be planktonic, non-planktonic or lecithotrophic, and this behaviour can last hours or even weeks (Sola, 1996).

The population has various individuals' sizes and their frequency depends on the reproductive season (Sola, 1996). As expected, after the breeding season a higher frequency of small individuals arises and the population biomass is higher (Sola, 1996).

2.3. Parasitism in *H. ulvae*

The endocrine disruption in *H. ulvae* can be caused by natural and/or artificial chemicals, yet they can be triggered either by abiotic factors, as suboptimal temperature or low pH, or biotic factors as food limitations or parasitism (Rato et al., 2009b). Despite be a part of the biological cycle of gastropods, parasitism by trematods is a natural factor rarely considered in such studies (Rato, 2009).

The parasitism impacts the hosts' ecology, modelling the animal communities and food chains (Fredensborg et al., 2006). In gastropods, it is a natural process, with greater prevalence in areas with bird populations.

The parasites life cycle includes both vertebrates and invertebrates hosts (Fig. 5). The eggs are produced in the gastrointestinal tract of birds and enter the ecosystem through their faeces (Fig. 5a) from which hatches a free-swimming miracidium that is ingested by the first intermediate host. This first host is usually a gastropod – a non-selective animal which passively ingests the miracidium. Inside the gastropod host there are several parasite metamorphoses, whose complexity is species-specific. In the first host a cercaria is produced, a larval form which is released into the aquatic system (Fig. 5b). Cercariae become encysted metacercariae on, or in, the second intermediate host – usually a crustacean. The metacercaria parasites the definitive host (Fig. 5c) – vertebrates – and it develops into the adult stage, which is capable of sexual reproduction (Fig. 5d). Thus, the life cycle of the parasitism is complete (Fredensborg et al., 2006; Mouritsen et al., 1997; Rato et al., 2009b).

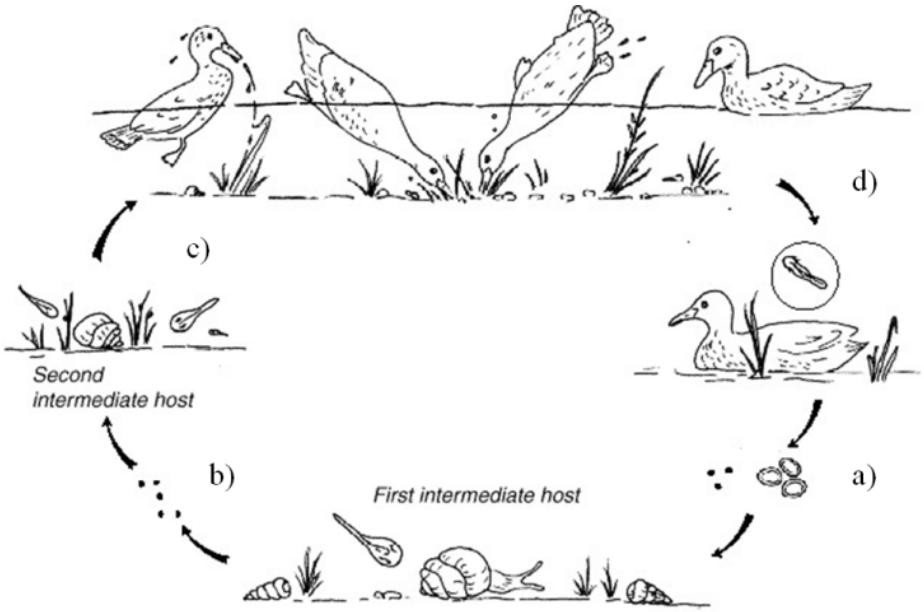

Figure 5 – Trematode life cycle: a) releasing of the eggs; b) releasing of cercaria; c) releasing of metacercaria; d) parasite' sexual reproduction. Adapted from Friend & Franson, 1991.

In 1934, the scientific community recognized the presence of parasitic trematodes in mud snails (Gorbushin, 1997). Trematodes inhabit gastropods' gonads and/or hepatopancreas, affecting their physiology by altering their immune and endocrine systems (Gorbushin, 1997; Rato et al., 2009b). Consequently, mud snails

may suffer behavioural changes, shell deformity, gigantism and tissue destruction, due to the energy depletion and, frequently, the destruction of the gonadal tissue. It may cause castration and/or alter the development of sex characteristics as absence of active sperm and reduced number of eggs (Field & Irwin, 1999; Gorbushin, 1997; Huxham et al., 1995; Rato et al., 2009b).

Gastropods' parasites are ubiquitous in all aquatic ecosystems and belong mostly to sub-class Digenea (Field & Irwin, 1999; Rato et al., 2009b). *Hydrobia ulvae* is the first intermediate host of six families of the sub-class Digenea: Echinostomatidae, Heterophyidae, Psilostomatidae, Notocotycidae, Haploporidae and Microphallidae (Field & Irwin, 1999). As explained above, the life cycle of the Digenea includes various hosts and life forms (Fig. 6), although the definitive hosts of *H. ulvae*'s trematodes are always shorebirds (Fredensborg et al., 2006).

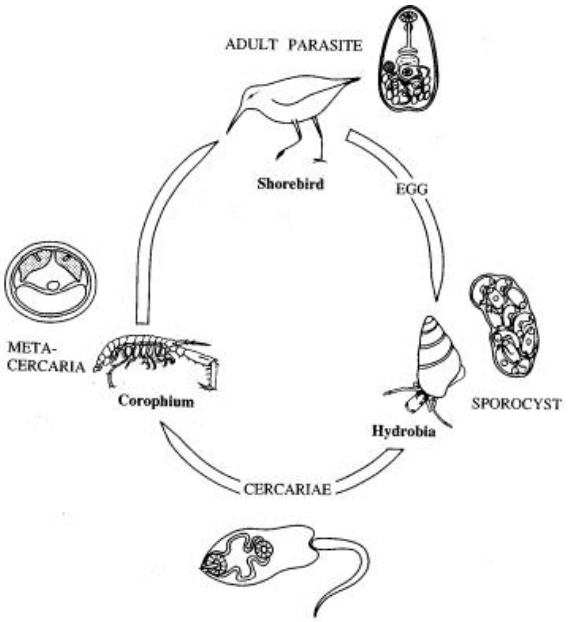

Figure 6 - Life cycle of digenean microphallid trematodes with intertidal invertebrates as first and secondary intermediate hosts, and shorebirds as final definitive ones. From Mouritsen et al. (1997).

Field & Irwin (1999) studied mud snails' populations, between January and December 1994, in the Ythan Estuary (North-East Scotland) and in Belfast Lough (Northern Ireland), and observed a higher prevalence of infected animals in the warmer months, especially in August. As the spatial and temporal distribution of parasites are dependent on the presence of hosts and favourable environmental conditions, the passive dispersion of *Hydrobia ulvae* influences the infection dynamics and the larvae spatial distribution (Field & Irwin, 1999; Fredensborg et al., 2006).

The consequences of the parasitic infection, under natural conditions, depend on four main factors: (i) the infection term; (ii) the infection intensity; (iii) the pathogenicity of the parasites; and (iv) the individual tolerance (Gorbushin, 1996).

Accordingly, the effects are higher, the greater are the infection term, intensity and parasites pathogenicity and lower is the specimen tolerance.

Several effects of *H. ulvae* parasitism are described as the infection by *Cryptocotyle sp*. that induces castration and gigantism, this later justified by the allocation of energy to the somatic growth: when compared to non-infected specimens, the sexually mature (castrated by the parasite) increase in size due to the redirection of the energy pool to somatic growth and not to gonads maturation. In fact, this effect is age-specific since juveniles are not affected: there are no significant differences on the animal size in sexually immature specimens since in this life stage much of the energy is used for somatic growth and not to reproduction (Gorbushin, 1996).

Other studies suggest that some parasites release toxic compounds into this gastropod bloodstream, reducing the gonads blood supply, and produce neurohormones that disrupt the mud snail's hormonal system, which may totally suppress their ovulation (Gorbushin, 1996). Moreover, infected males could show absence of active sperm cells and no copulatory organ or vas deferens while infected females might present an accentuated decrease in the number of eggs, sometimes zero, and also the occurrence of small non-functional penises, when compared to non-infected females (Gorbushin, 1997; Krull (1935) and Rothschild (1938) *in* Schulte-Oehlmann et al., 1997).

Therefore, *Hydrobia ulvae* females' masculinization is also trigged by parasitism and was registered prior 1960 (Schulte-Oehlmann et al., 1997), and so well before to the *imposex* first record in 1970 (Blabler (1970) *in* Smith, 1981). Even so, parasitism is not the only cause for *imposex* occurrence in this species, since it was already proven that *imposex*-affected females may or may not be parasitized (Galante-Oliveira et al., 2010a; Schulte-Oehlmann et al., 1997).

2.4. *H. ulvae* as a bioindicator species of TBT pollution

The first indication that *Hydrobia ulvae* could be used as a bioindicator of *imposex* was given by Schulte-Oehlmann et al. (1997), authors that described the phenomenon evolution in the species. Later, the same working team (Schulte-Oehlmann et al., 1998) established the TBT concentration from which *imposex* phenomenon is initiated in the mud snail: 20 ng TBT-Sn.l^{-1} in water and 20 ng TBT-Sn.g^{-1} (dry weight) in sediments.

Initially the following indices were suggested for the *imposex* assessment in *H. ulvae*: PL, RPLI, %I and VDSI, (Schulte-Oehlmann et al., 1997; see section 1.2, sub-section *Indices* for description). Some authors support that no variation on male penis length occurs during sexual cycle (Schulte-Oehlmann et al., 1997; Silva, 2002), which keeps RPLI a reliable index. Although, other factors are involved and a specific study on the effects of those factors on the different indices reliability prompting that: (i) despite narcotization facilitates animal handling and reduces penis measurement variance (since this organ ceases to contract or relax when the animal is anesthetized), it results in an increase of the penis length (by the tissues relaxation) thus affecting PL and RPLI values; and (ii) the size of *H. ulvae* selected for monitoring programmes has a major influence on the results, since smaller females (shell height <4.00mm) cause an underestimation of FPL, %I and VDSI, whilst variation in males size cause oscillations in RPLI, regardless of TBT pollution levels (Galante-Olivieira et al., 2010a). Therefore, the most reliable indices to be used for *imposex* assessment in this species are %I and VDSI, selecting (whenever possible) adult females with shell height > 4.00mm.

The vas deferens sequence (VDS) classification scheme used for *H. ulvae* (Fig. 7) is distinct from the one proposed by Gibbs et al. (1987) for *Nucella lapillus*. The VDS development in this species is classified in 5 stages: from the 'Stage 0' that represents a normal female to 'Stage 4' symbolizing a female with a vas deferens completely developed. In addition, as *H. ulvae* females show morphological variability of their female genital opening (i.e. either open or closed, regardless of their *imposex* level and/or their parasitized stage), the value 5 is added to the *imposex* stage when

females present the genital opening closed, resulting in 5 additional stages (from 5 to 9), (Schulte-Oehlmann et al. 1997). Beyond this variation, *imposex* development in *H. ulvae* is also classified into two pathways (Table 1): a '***a-type'*** where there is an additional growth of a penis in females and, in the course of the process, a vas deferens (see Fig. 7 stage 3*a* and Table 1) is formed; and a '***b-type'*** in which a vas deferens grows from the female orifice of the vaginal channel along the female head, being followed by the formation of a penis (see Fig. 7 stage 3*b* and Table 1).

Finally, Schulte-Oehlmann et al. (1998) suggested that the number of specimens analysed per sample should be defined according to the *imposex* levels registered at each site. At highly contaminated populations, with ≈ 80% of affected females, a minimum of 30 individuals should be observed (identical to the proposed for *N. lapillus*; Oehlmann (1994) in Schulte-Oehlmann et al., 1998) and at less contaminated sites (≈ 10% of affected females) at least 80 specimens should be analysed, since the indices values variability increases as *imposex* expression decreases thus affecting results significance.

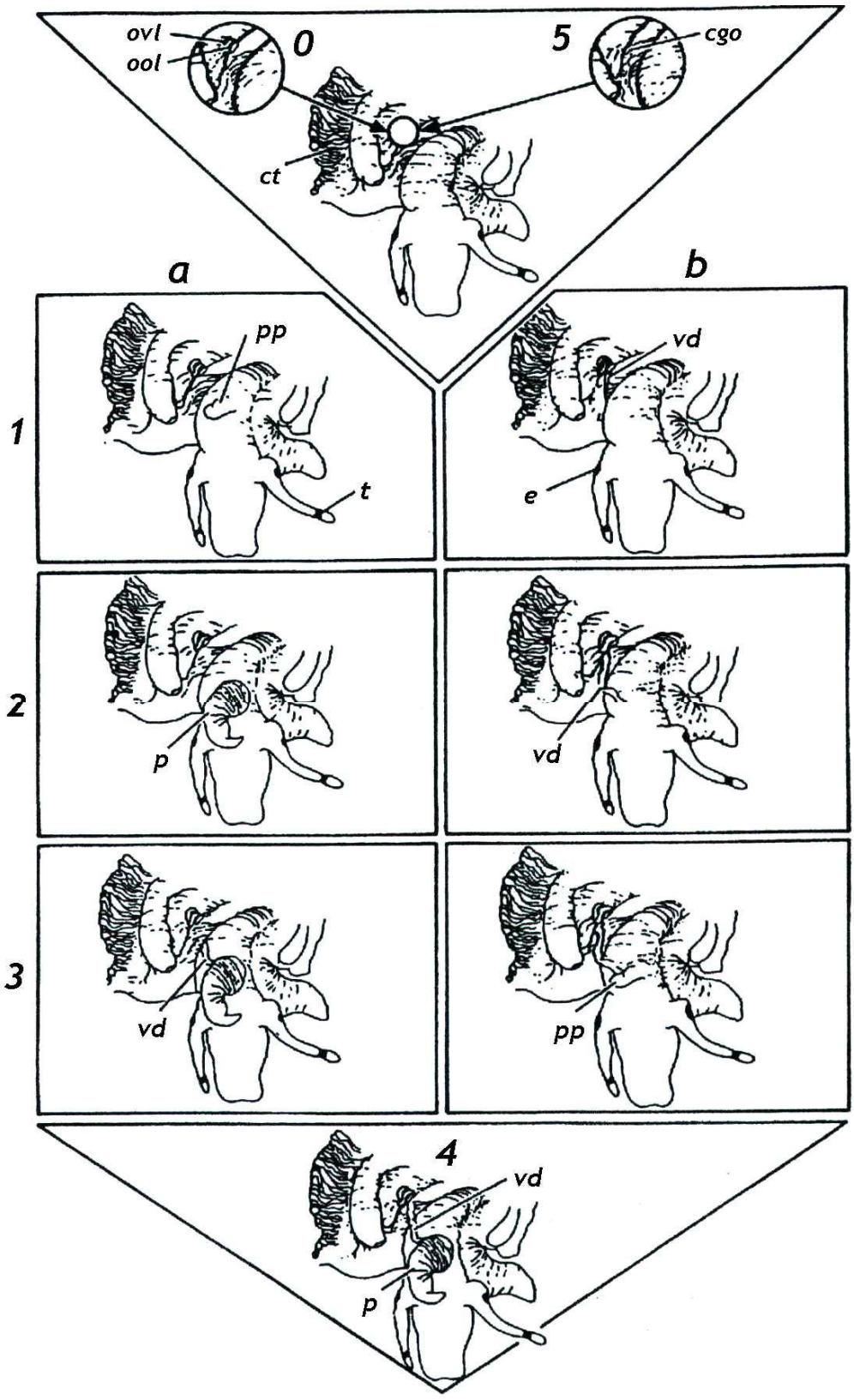

Figure 7 - *Hydrobia ulvae*. *Imposex* development scheme with five different stages. Stage 5 represents a sterilized female (genital orifice closed) without male characteristics: ovl – orifice of the vaginal channel; ool – orifice of the oviparous channel; cgo – closed genital orifice; pp - penis primordium; vd – vas deferens; p – penis. From Schulte-Oehlmann et al. (1997).

Table 1 – *Hydrobia ulvae*. Description of each *imposex* stage (1 to 4, compare Fig. 7) by the development type (*a*-type- and *b*-type), (Schulte-Oehlmann et al., 1997).

Stage	*a*-type	*b*-type
1	Development of a penis primordium without spermatic duct.	Formation of a gutter-like depression in front of the vulva.
2	Increased size of the penis and formation of the spermatic duct.	Formation of the vas deferens through structural modifications
3	Emergence of a distal section of the vas deferens at the base of the penis, which grows towards the genital orifice.	Formation of a penis primordium without spermatic duct.
4	Completion of the development of the vas deferens, which reaches the orifice of the female genital. The penis presents spermatic duct - conclusion of the *imposex* development by *b*- type.	

3. Ria de Aveiro

3.1. General description

Ria de Aveiro is a shallow coastal lagoon that can be classified as a bar-built estuary. It is located in the Portuguese Northwest coast, extending from from Ovar to Mira, reaching a maximum of 45 km parallel to the coast and 10 km wide (Barroso et al., 2002; Galante-Oliveira et al., 2010a; Pastorinho et al., 2011).

The average depth is 1 m and the maximum is about 4 m, although some areas are artificially maintained to increase its depth as the port areas and the system embouchure, with 7 and 20 m average depth respectively (Dias et al., 2001; Lopes et al., 2007).

The water transfer between the Atlantic Ocean and Ria de Aveiro is accomplished through the narrow lagoon mouth, which controls the hydrographical circulation (Barroso et al., 2000; Dias, 2001). The total area of the Ria de Aveiro is

dependent on the tides: on high tide, Ria de Aveiro covers 47 km^2 and on low tide, 43 km^2 (Barroso et al., 2000).

Ria de Aveiro is composed by 4 main channels: S. Jacinto, Espinheiro (northern channels), Mira and Ílhavo (southern channels). Several local rivers flow into Ria de Aveiro as the Vouga, Boco, Caster and Antuã rivers (Fig. 8; Barroso et al., 2000; Pastorinho et al., 2011).

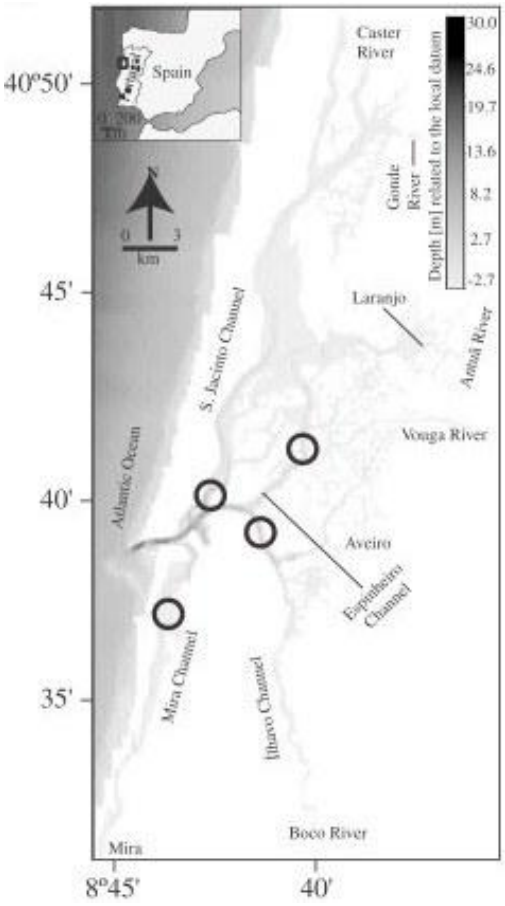

Figure 8 – Schematic representation of the Ria of Aveiro with the main rivers and channels. Adapted from Vaz et al. (2009).

The tidal action and salinity decreases progressively along the channels as both are dependent on the distance from the system mouth. Salinity presents longitudinal gradients, but this depends either on rainfall or tidal phase (Barroso el al., 2000). Ílhavo and Mira channels present higher salinity values than the S. Jacinto channel: during the rainy season, salinity in the southern channels vary between 0-4 (Ílhavo channel) and 0-2 (Mira channel) while in the dry season 2-9 and 4-11, respectively; in the northern channel, salinity varies between 7 and 25 psu throughout the year (Barroso et al., 2000).

Along the Ria de Aveiro estuarine system there are several branches, narrow islands, mud and sand banks and salt marshes, which are habitats for several organisms' *taxa* (Silva, 2002) being inclusively classified as a special protected area by the European Union Nature and Biodiversity Policy since 2000 (Galante-Oliveira et al., 2010a). However, it covers several activities that support the local economy, such as aquaculture, fisheries and maritime transport, increasing the risks associated with pollution namely by TBT compounds (Barroso et al., 2000; Galante-Oliveira et al., 2010a; Pastorinho et al., 2011; Sousa et al., 2009a).

3.2. TBT pollution sources in Ria de Aveiro

The excessive use of anti-fouling (AF) paints during decades, in vessels and aquaculture equipment, led to the release of vast quantities of TBT compounds into aquatic ecosystems, either by leaching or by the removal of old TBT-based AF systems in dockyards (Batley, 1996). Thus, marinas, dockyards and areas with high naval traffic have been described as the main sources of TBT compounds to the aquatic environment (Batley, 1996). Similarly, the main hotspots of TBT pollution, that have been described in Ria de Aveiro, are: the most busiest port terminals, the dockyards, smaller maritime infrastructures such as marinas and artisanal fishing docks, and also the main navigation channels (Barroso et al., 2000; Galante-Oliveira et al., 2009).

In 1998, 85% of the Ria de Aveiro vessel fleet, which was mainly constituted by both large commercial and fishing ships, used TBT-based AF paints in their hulls (Barroso et al., 2000). Additionally, TBT inputs in this estuarine system were also promoted by the construction and repair dockyards, specialized in vessels larger than 25 m, in which about 70% of the paints applied were TBT-based (Barroso et al., 2000). These compounds release from all these sources during decades, and its adsorption to sediments with low degrading rates, implied that sediments have been acting as reservoirs, carrying on their slowly release (Langston & Pope, 1995; Burton et al., 2006; Ruiz et al., 2008).

Therefore, as established in 1998 by Barroso et al. (2000), TBT pollution monitoring in Ria de Aveiro includes the sampling of the following sources (Fig. 9):

(i) Five port terminals, located along the main navigation channel (i.e. that connects the system embouchure and the city of Aveiro): a) both north and south commercial terminals (Fig. 9C and 9F); b) the chemical terminal (Fig. 9B); c) the deep-sea fishing port (Fig. 9E); and d) the coastal fishing port (Fig. 9A), near the city of Aveiro.

(ii) The main dockyard, that is still located at the north top of the Ílhavo Channel (Fig. 9E);

(iii) The three main marinas (Costa Nova marina, Fig. 9H; Gafanha marina, Fig. 9I; and Ovar marina, Fig. 9K), that also hosts some of the numerous small fishing and pleasure boats that are spread throughout the Ria, mainly in the channel that crosses the city of Aveiro, in the Mira Channel and at Torreira and Murtosa villages.

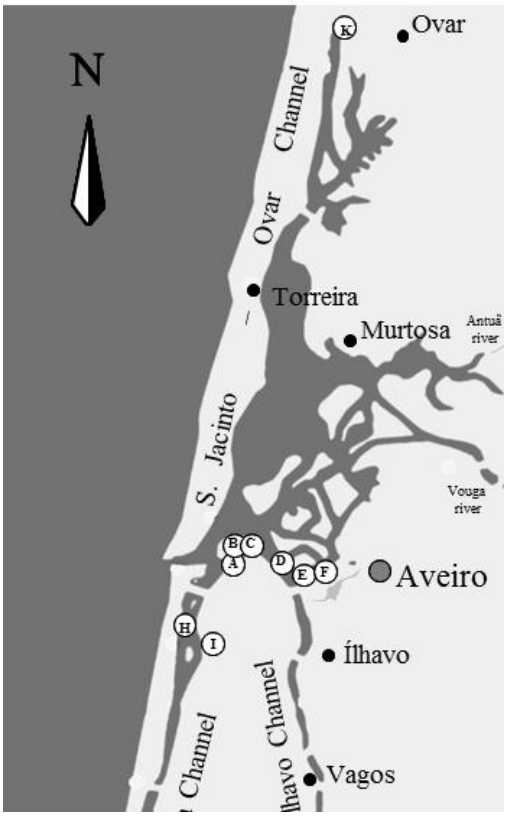

Figure 9 – Ria de Aveiro, showing the main TBT contamination sources. A – Coastal fishing port; B- Chemical port; C – North commercial port; D – Deep-sea fishing port; E – Aveiro dockyards; F – South commercial port; H – Costa Nova marina; I – Gafanha marina, and K – Ovar marina. Adapted from Barroso et al. (2000).

3.3. Biomonitoring of TBT pollution in Ria de Aveiro

The first study regarding the TBT pollution impact in the Ria de Aveiro was conducted in 1998 by Barroso et al. (2000). These authors sampled four species of marine prosobranch gastropods that were already validated as indicators of *imposex* levels: *Nucella lapillus* (Bryan et al., 1986; Gibbs et al., 1987; Gibbs & Bryan, 1996), *Nassarius reticulatus* (Stroben et al., 1992; Bryan et al., 1993), *Littorina littorea* (Bauer et al., 1995, 1997) and *Hydrobia ulvae* (Schulte-Oehlmann et al., 1997, 1998). Since they present complementary distribution, these species combined use allowed biomonitoring TBT pollution in the entire estuarine system, including the TBT main sources (Barroso et al., 2000; for these sources description see the above section 3.2). Beyond that, these species showed distinct sensibility to TBT compounds: *N. lapillus*

was the most sensitive, corroborating the data on the *imposex* initialization at very low TBT concentrations in water (<0.5 ng TBT-Sn.l^{-1}; Gibbs & Bryan, 1986), followed by *N. reticulatus*, *H. ulvae* and *L. littorea*. At that time, *N. lapillus* was useful to monitor *imposex* levels at the coastline but was not a good bioindicator to disclose different levels of *imposex* between the most polluted sites and, therefore, Barroso et al. (2000) proposed the combined use of *N. reticulatus* and *H. ulvae* for the TBT pollution monitoring of estuarine areas moderately to highly polluted by TBT compounds, such as Ria de Aveiro.

Three years after Barroso et al. (2000) publication, the definite EU ban on the use of TBT-based AF paints by the EC Regulation 782/2003 was implemented (see section 1.1 sub-section *Regulating the use of TBT*). It was therefore of crucial importance to create baselines of pollution levels by TBT at the time of that measure entry into force, for the posterior evaluation of its efficacy. Hence, several biomonitoring studies emerged to monitor TBT pollution in the Portuguese coast, including sites in Ria de Aveiro region, using different bioindicators: *N. lapillus* was sampled in 1997, 2000, 2003 and every year from 2005 to 2009 (Barroso & Moreira, 2002; Galante-Oliveira et al., 2006; Galante-Oliveira et al., 2009; Galante-Oliveira et al., 2010b; Galante-Oliveira et al., 2011; Laranjeiro et al., 2010); *N. reticulatus* was collected every year from 1997 to 2000, 2002 to 2006, and 2008 to 2010 (Barroso et al., 2002; Barroso et al., 2005; Barroso et al., 2011; Laranjeiro et al., 2010; Rato et al., 2006; Rato et al., 2009a; Rato et al., 2009b; Sousa et al., 2005; Sousa et al., 2007; Sousa et al., 2009a); *Mytilus galloprovincialis* in 2000, 2005 and 2006 (Barroso et al., 2004; Sousa et al., 2007; Sousa et al., 2009b); and *H. ulvae* in 2003, 2004 and 2007 (Galante-Oliveira et al., 2010a). All these studies included sampling sites in Ria de Aveiro at one or more of the following areas: coastline, inside the estuary and the offshore in front of the system (see a summary on Table 2).

Table 2 – Summary of the TBT pollution biomonitoring data available in the literature and that include sampling sites in Ria de Aveiro. All studies found refer to campaigns or along the Portuguese coast (Portuguese coastline) or in Ria de Aveiro (inside the estuarine system, along the estuary coastline and offshore) and used *Nucella lapillus*, *Mytilus galloprovincialis*, *Nassarius reticulatus* and *Hydrobia ulvae* as bioindicators. The years in which sampling occurred (and therefore with which samples are referred) are indicated in bold.

	Ria de Aveiro			**Portuguese Coastline**
	INSIDE	COASTLINE	OFFSHORE	
Nucella lapillus	**1997, 2003, 2005, 2006 and 2007** (Galante-Oliveira et al., 2009) **2009** (Laranjeiro et al., 2010)			**2000** (Barroso et al., 2002) **2003** (Galante-Oliveira et al., 2006) **2003, 2006 and 2008** (Galante-Oliveira et al., 2011)
Mytilus galloprovincialis	**2005** (Sousa et al., 2007)			**2000** (Barroso et al., 2004) **2006** (Sousa et al., 2009b)
Nassarius reticulatus		**1997; 1998 and 1999** (Barroso et al., 2005) **2002; 2004 and 2005** (Rato et al., 2006) **2005** (Sousa et al., 2007)	**2006 and 2010** (Barroso et al., 2011)	**2000** (Barroso et al., 2002) **2006** (Rato et al., 2009a;b) **2003** (Sousa et al., 2005) **2008** (Sousa et al., 2009a)
	2009 (Laranjeiro et al., 2010)			
Hydrobia ulvae	**2003; 2004 and 2007** (Galante-Oliveira et al., 2010a)			

N. lapillus was widely used to evaluate TBT pollution at coastal areas, even since it was the bioindicator recommended by the OSPAR JAMP guidelines for the NE Atlantic shores (OSPAR Ref. No: 2003-10). However the species inhabits marine rocky shores, having limited distribution in Ria de Aveiro (occurs only at the coastline and inside the estuary is restricted to some rocky artificial banks at the outer part of the navigation channel; Barroso et al., 2000). The species was used along the Portuguese NW coast and specifically in Ria de Aveiro, by Galante-Oliveira et al. (2009), to evaluate the spatio-temporal evolution at two sites inside and other two outside the system.

Regarding *Mytilus galloprovincialis*, the greater advantage is that, as a filter feeding, by analysing the TBT body burden is possible to calculate the expected concentration of these compounds in the water by the application of a bioconcentration factor (Takahashi et al., 1999b; Sousa et al., 2007). In 2005, Sousa et al. (2007) performed an integrative assessment of TBT pollution in the entire estuary, evaluating all ecosystem compartments (biota, sediments and water) by using this species and analysing TBT concentrations in tissues and sediments. Despite the wide geographic distribution, the chemical analysis of this species' tissues does not allow the direct observation of the biological effects of the pollution by TBT compounds, as it is noticed by *imposex* levels assessment.

Nassarius reticulatus is, by far, the most used species to monitor TBT pollution in Ria de Aveiro (see Table 2). It is also recommended by OSPAR to monitor areas where *Nucella lapillus* is not available, is widely distributed and very abundant, both inside the Ria de Aveiro, factors that contributed for its success as bioindicator. Beyond the simple record of pollution levels, this species allowed the gradients characterization, both of ship density inside the Ria (Barroso et al., 2005; Rato et al., 2006; Sousa et al., 2007) and the inshore-offshore (Rato et al., 2006), i.e. the TBT pollution sources identification and the monitoring of deeper areas. The greatest limitation is its absence from the channels inner areas, with very low salinity values, sites where *H. ulvae* is present and of crucial importance.

Hydrobia ulvae is an ubiquitous species in Ria de Aveiro and its with a local lifespan was estimated in approximately of 2 years (Silva, 2002), which is particularly relevant when evaluating pollution declining as a consequence of legislative action (i.e. since *imposex* is largely irreversible and its reduction can only be registered when older, and most affected, specimens are substituted by younger ones; Gibbs, 1999). However, the temporal evolution of *imposex* levels assessed in 1998 (Barroso et al., 2000), 2003, 2004 and 2007 (Galante-Oliveira et al., 2010a) contrasts with what has happened with other indicator species in the same area for the same period, namely in *Nucella lapillus* (Galante-Oliveira et al., 2009) and *Nassarius reticulatus* (Barroso et al., 2011). Galante-Oliveira et al. (2010a) point this result as an indication of TBT pollution persistence in sediments and suggest the sediment ingestion as feeding

habit in *H. ulvae* as the reason to choose this species as a bioindicator of TBT pollution persistence in this ecosystem compartment.

4. Objectives

The present study aims to assess the current status of TBT pollution and its temporal evolution during the last 15 years in Ria de Aveiro estuarine system, specifically regarding these compounds persistence in sediments. *Hydrobia ulvae imposex* expression was the selected biomarker as this species has been pointed as a bioindicator of TBT pollution persistence in sediments. In addition to the biomarker assessment, sediments analysis was also performed and correlated with the *imposex* intensity.

II. Material and Methods

1. Sampling

Adult specimens were randomly collected, by hand at the intertidal during low tide, between February and June 2012 at 10 sampling sites along Ria de Aveiro (Fig. 10). Whenever available, a minimum of 60 animals were collected at each site (St.). At St. 1 and 3 (see Fig. 10) larger samples were obtained in order to study the effect of the animals' size on *imposex* levels. Therefore, specimens of all available sizes from each population were collected. Once in the laboratory, animals were maintained in constantly aerated artificial seawater (Ocean Fish) at 19±1^{0}C and 25±1 psu until analysis.

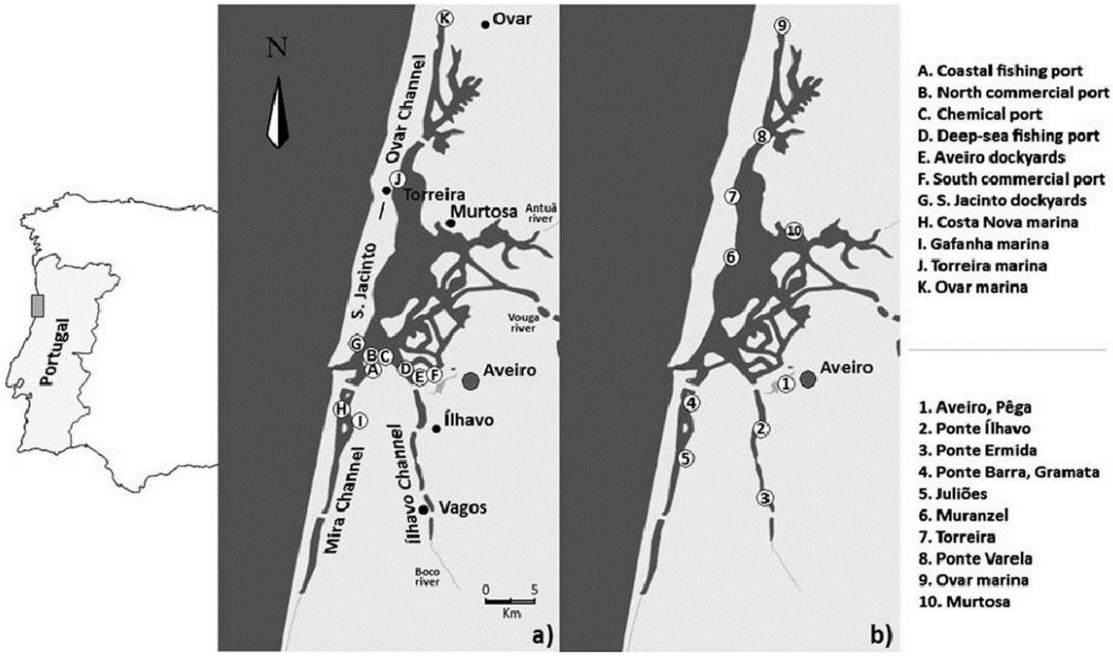

Figure 10 - Ria de Aveiro and adjacent coastal area map indicating: a) potential TBT pollution sources represented by ports, dockyards and marinas (A–K); b) sampling sites location, code and name (1–10). From Galante-Oliveira et al., 2010a.

Additionally, surface sediments (± 1 cm depth) were also collected at each sampling site, transported to the laboratory under cold conditions (c.a. 500 g wet weight in polyethylene bags) and frozen at –20°C until further processing.

2. Organotins (OTs) analysis in sediments

Organotin compounds were quantified in whole sediment samples according to the method described by Sousa et al. (2012). Briefly, deuterated labelled standards (d_9-MBT, d_{18}-DBT, d_{27}-TBT, d_{10}-DPT, d_{15}-TPT, d_{17}-MOT, d_{34}-DOT) were spiked into 2.5 g dry weight (dw) of freeze-dried sample with 1-2 g of ascorbic acid with ethylacetate. Organotins (OTs) in the samples were extracted by 1N HBr/methanol-ethyl acetate (1:1) by ultra-sonication and then transferred into ethylacetate/hexane (3:2) and concentrated by rotary evaporation. OTs in the extract were ethylated by adding 1mL of 5% tetraethyl sodium borate. After ethylation, the extract was cleaned up by 1M KOH and SEP-PAK plus florisil cartridge (Waters). OTs were eluted by 5% diethylether/hexane and then solutions were concentrated into 1 mL and spiked with 50 ng of deuterated tetrabutyltin used as a recovery standard. The final solutions were injected into a gas chromatograph-mass spectrometric detector (GC-MSD; Hewlett-Packard 6870 GC system with 5973 mass selective detector and 7683 series auto sampler). OTs were measured by GC-MSD in selected ion monitoring mode (EI-SIM) and quantified by isotope dilution method. To assess the QA/QC of measurements, the NIES CRM No.12 certified sediment reference material was analysed by the same method. Results obtained from 3 replicates analysis (0.19±0.001 μg g^{-1} for TBT and 0.009±0.001 μg g^{-1} for TPT, both in dw basis as chloride foams) are in accordance with the certified value reported for TBT (0.19±0.03 μg g^{-1}) and with the reference value for TPT (0.008 μg g^{-1}). In addition, a procedural blank was included with each analytical batch, to check for interfering compounds and to correct sample values, if necessary. Methods gave a tin detection limit (in terms of ng Sn g^{-1} dw) of 0.1 for MBT; 0.4 for DBT; 0.1 for TBT; 0.1 for DPT; 0.1 for TPT; 4.3 for MOT and 1.0 for DOT. All chemicals (for ultratrace analysis) were purchased from Wako Pure Chemical Industries (Japan), except hydrobromic acid (Kanto Chemical Co., Inc., Japan) and sodium tetraethylborate (Hayashi Pure Chemical Ind., Co., Ltd., Japan). Standard solutions were purchased from Hayashi Pure Chemical Ind., Co., Ltd., Japan.

3. Specimens analysis and *imposex* characterization

Individuals were narcotized in Magnesium Chloride (MgCl) 3.5% in distilled water for about 60 minutes, as recommended by Galante-Oliveira et al. (2010a). Shell height (SH) was then measured, under a stereo microscope (OLYMPUS SZH10) equipped with a graduated eyepiece, to the nearest 0.14 mm (Fig. 11a). At St. 1 and 3 (selected to study the effect of the animals' size in the *imposex* levels) specimens were measured and separated in SH classes, defined as indicated in Table 3. Whenever available, 15 females and 10 males were analysed per SH class. In the remaining sites (St. 2, 4-10), and whenever possible, 40 adult females and 20 adult males were analysed for *imposex* as follows.

Shells were cracked with a bench vice and removed. Then, specimens were dissected (Fig. 11b), sexed and analysed for *imposex*: the penis length (PL) measured under the stereo microscope to the nearest 0.06 mm and the Vas Deferens Sequence (VDS) stage determined in females (Fig. 11c) according to scoring system developed by Schulte-Oehlmann et al. (1997). The presence of parasites was carefully checked and, although *imposex* levels in parasitized specimens were also assessed to investigate the correlation between parasitism and female masculinisation, these specimens were not included in *imposex* indices calculation, as recommended by Schulte-Oehlmann et al. (1997, 1998).

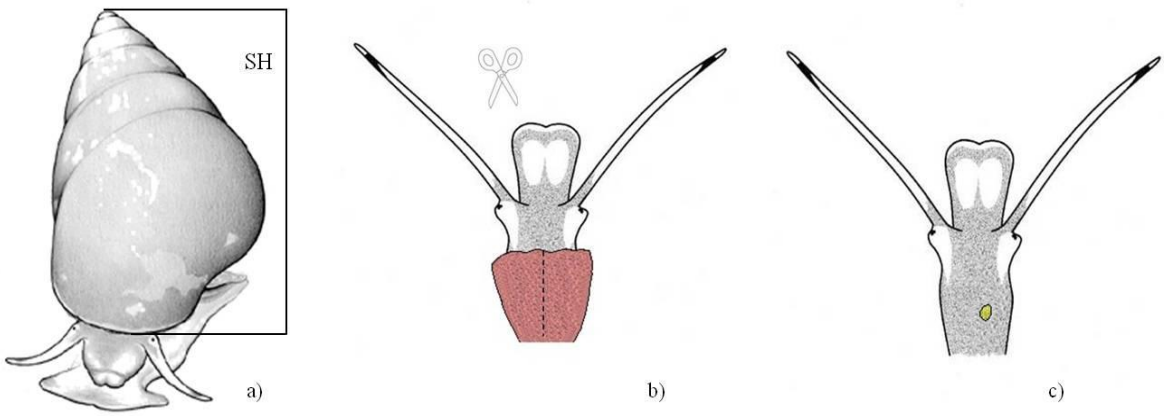

Figure 11 – *Hydrobia ulvae*. Schematic representation of *imposex* analysis: a) measurement of shell height (SH); b) Mantle (pink region) cutting (dashed line through the mantle region); c) identification of a masculinized female (*a* type *imposex*, stage 1; at yellow shaded is represented a penis primordium). Adapted from http://www.habitas.org.uk/.

Table 3 – *Hydrobia ulvae*. Shell height classes (respective number and interval) defined to study the influence of the animals' size on the mud snail *imposex* levels.

Class	Shell Height (mm)
1	1.50 - 2.75
2	2.76 - 4.00
3	4.01 - 5.25
4	5.26 - 6.50
5	6.51 - 7.75

The following *imposex* indices were calculated: the percentage of *Imposex*-affected Females (%I), the Mean Female Penis Length (FPL), the Mean Male Penis Length (MPL), the Vas Deferens Sequence Index (VDSI) and the Relative Penis Length Index (RPLI) calculated by $\frac{\text{Mean Female Penis Length}}{\text{Mean Male Penis Length}} \times 100$. At St. 1 and 3, these indices were calculated by SH class while for the remaining (St. 2, 4-10) were per site (i.e. population).

4. Organic matter quantification

The organic matter content of the sediment was estimated from the loss weight. Sediment was pre-dried at 58º C for 72 hours, into a ceramic crucible and ignited inside a muffle furnace for 5 h, at 550ºC.

5. *Hydrobia ulvae* annual cycle of gonads maturation

The results concerning the effect of sexual maturation on *imposex* levels in *H. ulvae* were originally obtained by Silva (2002) at Ermida (St. 3, see Fig. 10) from December 1999 to December 2000. However, all the data were revised for this study, including a complete reclassification of the gametogenic stages from 15 females and 15 males, which required a reanalysis of all the gonad histological preparations that

were preserved for almost a decade. Three slides with 5 sections each were available by specimen and were observed under light microscopy (OLYMPUS BX40) in order to determine the individual gametogenesis stage using the six stages scale (see Table 4) – I (immature), II (early recovering), III (late recovering), IV (ripe), V (partially spent) and VI (spent) – proposed by Barroso and Moreira (1998) for prosobranch gastropods and already used by other authors and other species (e.g. in *Nucella lapillus* by Galante-Oliveira et al., 2010b). Considering that varying proportions of all maturity stages could be observed throughout the year, and whenever possible, three follicles from each gonad section were examined (3 follicles x 5 sections x 3 slides = 45 observed follicles) and the individual gametogenesis stage considered was the mode value amongst those registered for the 45 observed follicles.

Table 4 – *Hydrobia ulvae*. Gametogenesis stages description. Adapted Barroso & Moreira (1998).

Stage	Females	Males
Stage I **Immature Cells**	The gonad was an internal structure not detailed and presents primordial germ cells enfolded in connective tissue.	
Stage II **Early Recovery**	Presence of separated follicles enfolded in connective tissue. Near of the follicle's wall occur the first's gametogenesis stages.	
Stage III **Late Recovery**	Follicles are closer and the connective tissue is lower. Oocyte reaches half of its growth.	Small concentrations of sperm appear in lumen
Stage IV **Ripe**	Disappearance of the connective tissue. Follicles are full and mature. Follicles are full with mature oocytes. It can contain oocytes on Stage III.	Follicles are distended and show sperm in the middle while spermatogenesis still occurring in the periphery.
Stage V **Partially Spent**	Existence of some connective tissue between the follicles, especially on females. Lumen became almost empty. Most follicles are mostly empty, although they contain some mature oocytes in the middle, while oogenesis is declining on the cell walls.	Most follicles are mostly empty although they contain some sperm in the middle, while spermatogenesis is declining on the cells walls.
Stage VI **Spent**	It varies from the initial phase of large empty follicles until a phase of tiny follicles scattered in connective tissue – it is on its maximum development.	

6. Statistical data analysis

Statistical comparisons of *imposex* levels between the different surveys [data of 2003, 2004 and 2007 surveys by Galante-Oliveira et al. (2010a)] were performed by regression analysis using the software R version 2.15.2 from 26 of October 2012 by R Foundation for Statistical Computin. Spearman correlations were performed using the software Statistica 6.0 from StaSoft, Inc 2002. Results were considered statistically significant for $P<0.05$.

III. Results

1. Spatial evolution of TBT sediment contamination and *H. ulvae imposex* in Ria de Aveiro - 2012 survey

1.1. Organotins (OTs) concentration in sediments

Results for the concentration of the organotins TBT, DBT and MBT in sediments are shown in Table 5. The levels of TBT varied from 1.5 to 29 ng Sn. g^{-1} (dw) whilst DBT ranged from 0.9 to 14 ng Sn. g^{-1} (dw) and MBT from <0.1 to 23 ng Sn. g^{-1} (dw). Triphenyltin, diphenyltin, dioctyltin and monoctyltin were always below the detection limits. The highest contamination by TBT was observed at Juliões (St. 5) and Ermida (St. 3), both representing the most upward sites sampled in the Mira and Ílhavo channels, respectively. In order to investigate the predominance of TBT over its metabolites, to assess the occurrence of fresh TBT inputs into surface sediments, the butyltin degradation index (BDI = (MBT + DBT)/TBT) proposed by Díez et al. (2002) was determined (Table 5). In this calculation the MBT values <0.1 were converted to 0.05. BDI varied between 0.3 and 2.4 indicating fresh inputs of TBT only at Sts. 4, 7, 8, 9.

The organic content of sediments at each station varied between 2.36 and 17.9 (% of dry weight (dw)), in St. 9 and 3, respectively (Table 5). There was a non-significant correlation between TBT concentration and the sediment organic matter content ($r=0.36$; $P>0.05$).

Table 5 - Organotins concentration (ng Sn. g^{-1} dw) and Organic matter content (%OM) in sediments from Ria de Aveiro in 2012 survey. Recent TBT inputs in sediments are assessed by BDI (Butyltin Degradation Index.

Sampling Site	MBT	DBT	TBT	BDI	OM
St. 1 (Pega)	8.2	4.5	5.2	2.4	5.9
St. 3 (Ermida)	15	5.4	11	1.9	18
St. 4 (Gramata)	<0.1	2.0	3.1	0.7	4.9
St. 5 (Juliões)	23	14	29	1.3	4.4
St. 7 (Torreira)	<0.1	0.9	1.5	0.7	4.7
St. 8 (Ponte Varela)	<0.1	1.0	3.2	0.3	6.7
St. 9 (Marina Ovar)	<0.1	1.6	2.8	0.6	2.4

1.2. *Hydrobia ulvae imposex* levels

Imposex levels of *Hydrobia ulvae* in Ria de Aveiro for the year 2012 are shown in Table 6 and Figure 13. The results refer only to non-parasitized females, as generally recommended for *imposex* studies when using *H. ulvae* or any other prosobranch species (Schulte-Oehlmann et al., 1997). In this context, the parasitized specimens were discarded from this analysis. The abundance of *H. ulvae* differed greatly between sampling sites and the same also happened regarding the prevalence of digenean parasitism, which attained values in the range of 0-78%. Hence, the total number of adult non-parasitized females that were used to estimate *imposex* parameters at each station varied between 30-40 females for most of the sites (Sts. 1, 3-8) and 6 females for Sts. 2 and 9. The average size of the females also varied across sites, with a minimum SH of 2.7 mm at St. 2 and a maximum of 6.0 mm at St. 7. Most of the *H. ulvae* females from Ria de Aveiro in 2012 are affected by *imposex* as %I ranged between 76 and 100. Females exhibit only VDS stages 0, 1 or 2 (always *a*-type) and VDSI presented relatively uniform levels throughout the study area, varying between a minimum of 0.93 at St. 8 and a maximum 1.17 at St. 5. Similarly, the female penis length (FPL) did not differ greatly within the study area, reaching a lowest value of 0.14 mm at St. 2 and a highest value of 0.41 at St. 1, which corresponds to 13-23% of the male penis length (RPL) throughout sampling sites. There is a high correlation

between VDSI and FPL across sites (r=0.92, P<0.01), which is expected since both parameters express the same phenomenon; for this reason graphs regarding FPL are omitted from Figure 13 to avoid data duplication. Correlation renders not significant between %I and VDSI (r=0.46, P>0.05) and between %I and FPL (r=0.24, P>0.05). Curiously, there is no significant correlation between the concentration of TBT in sediments and any of the *imposex* parameters across the seven sampling sites listed in Table 4 (TBT *vs* %I, r=-0.04; TBT *vs* VDSI, r=0.52; TBT *vs* FPL, r=0.57).

Table 6– *Hydrobia ulvae*. Analysis of *imposex* parameters in non-parasitized females per sampling site, with the indication of their geographic coordinates. The *imposex* parameters are represented by the vas deferens sequence index (VDSI), mean female penis length (FPL), percentage of *imposex* affected females (%I) and relative penis length index (RPLI), with an indication of the number of females (N ♀) and males analysed (N ♂). The percentage of parasitized females and males (%P) for each sampling site is also indicated. SH - female mean shell height.

Sampling Site			***Imposex* parameters**						**Parasitism**	
St. Code	**Name**	**Coordinates**	**N ♀**	**SH**	**VDSI**	**FPL**	**% I**	**RPL (N ♂)**	**% ♀**	**% ♀**
1	Pega	40°38'2.60"N 8°39'41.21"W	37	5.60	1.05	0.41	89	23.1 (29)	20	20
2	Ponte Ilhavo	40°36'25.89"N 8°41'3.98"W	6	2.73	1.00	0.14	100	17.0 (10)	0	0
3	Ermida	40°34'22.44"N 8°40'51.18"W	30	5.15	1.03	0.37	100	23.0 (16)	10	10
4	Gramata	40°37'31.13"N 8°44'10.19"W	61	5.09	0.95	0.25	93	14.9 (20)	33	23
5	Juliões	40°37'15.83"N 8°44'15.42"W	61	5.96	1.17	0.38	100	21.5 (20)	33	38
6	Muranzel	40°40'20.47"N 8°43'21.24"W	41	4.20	1.00	0.21	98	13.3 (21)	2	0
7	Torreira	40°45'8.84"N 8°42'5.97"W	43	6.00	1.03	0.34	100	17.1 (20)	7	0
8	Ponte Varela	40°47'26.89"N 8°40'28.04"W	58	5.60	0.93	0.27	76	14.7 (20)	31	29
9	Marina Ovar	40°51'34.31"N 8°39'19.18"W	27	4.99	1.00	0.30	100	20.27 (6)	78	73

2. Effect of *H. ulvae* size, parasitism and TBT sediment contamination on female *imposex* levels and male penis

2.1. Effect of animal size, parasitism and TBT sediment contamination on *imposex*

In order to better interpret the effect of the female size, parasitism and TBT environmental contamination on the individual levels of *imposex* in *H. ulvae*, a regression analysis must be performed considering all specimens of the sampling sites for which there is available data regarding all these factors. For this purpose, VDS and FPL *imposex* data of all females (parasitized and non-parasitized) from Sts. 4, 7, 8 and 9 (N=189) are analysed using two different regression models depending on the nature of the *imposex* variables. VDS is an ordinal variable that requires an ordered logit regression model (OL), whilst FPL is an interval-scale variable for which an ordinary least squares regression (OLS) is appropriate. The results reveal a significant and positive effect of SH and parasitism on the levels of VDS, but TBT has no significant effect on this variable (Table 7). Similarly, SH and parasitism have a highly significant positive effect on FPL whilst there is no significant effect regarding TBT (Table 8).

Table 7 - Results of the latent variable logit model (ordered logit model) for the ordinal variable Vas Deferens Sequence (VDS). VDS*: latent continuous variable of the ordinal VDS; β_0, β_1, β_2: coefficients for each parameter; SH: shell height; Par: female with or without parasites; TBT: concentration of TBT in sediments; ε: associated error. N=189 females.

$VDS^* = \beta_0 SH + \beta_1 Par + \beta_2 TBT + \varepsilon$

Coefficients	Value	*t*-value	*p*-value	
Intercepts:				
0|1	-0.501	-0.298	7.7×10^{-1}	n.s.
1|2	5.662	3.256	1.1×10^{-3}	**
Parameters:				
SH	0.491	2.196	2.8×10^{-2}	*
Par	1.856	3.185	1.4×10^{-3}	**
TBT	-0.183	-0.498	6.2×10^{-1}	n.s.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s.: not significant.

Table 8 - Linear least squares regression results for the variable female penis length (FPL). β_0, β_1, β_2; β_3: coefficients for each parameter; SH: shell height; Par: female with or without parasites; TBT: concentration of TBT in sediments; ε: associated error. Ajusted R^2 = 0.26. F-statistic = 23.4 (185 degrees of freedom; P = 6.7 x 10^{-13}); N=189 females.

FPL = β_0 + β_1 SH + β_2 Par + β_3 TBT + ε

Coefficients	Value	*t*-value	*p*-value	
Intercept	-0.218	-2.010	4.6 x 10^{-2}	*
SH	0.091	6.169	4.2 x 10^{-9}	***
Par	0.120	4.021	8.4 x 10^{-5}	***
TBT	0.000	0.011	9.9 x 10^{-1}	n.s.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s.: not significant.

Since parasitism has a significant positive impact on *imposex*, this aspect deserves a deeper insight through historical data regarding the relationship between *imposex* and parasitism. Table 9 shows the prevalence of parasitism on *imposex* affected females and normal females. The parasites were classified as digeneans but none has been identified to species so far. Remarkably, in all surveys performed in Ria de Aveiro from 2003 to 2012 parasitism was only detected in *imposex* affected females.

Table 9 – *Hydrobia ulvae*. The total number (*n*) of specimens observed in 2003, 2004, 2007 (Galante-Oliveira et al., 2010a) and 2012 (present study) surveys and the corresponding percentage of parasitized females (P%) is indicated per year and sampling site (St.) for: females affected with *imposex* (♀ VDSI ≥ 1) and unaffected females (♀ VDSI = 0). n.d.: no data available.

		♀ VDSI ≥ 1		**♀ VDSI = 0**	
St.	**Year**	***n***	**P %**	***n***	**P %**
1 Pega	2003	28	7.1	22	0.0
	2004	30	0.0	22	0.0
	2007	42	19.5	4	0.0
	2012	54	n.d.	9	n.d.
2 Ponte de Ílhavo	2003	29	0.0	11	0.0
	2004	25	0.0	8	0.0
	2007	34	8.8	5	0.0
	2012	6	0.0	0	0.0
3 Ermida	2003	34	0.0	25	0.0
	2004	34	2.9	8	0.0
	2007	36	5.6	5	0.0
	2012	59	n.d.	1	n.d.
4 Gramata	2003	18	0.0	25	0.0
	2004	47	59.6	8	0.0
	2007	45	42.2	17	0,0
	2012	41	34.0	3	0.0
5 Juliões	2003	21	0.0	19	0.0
	2004	23	0.0	17	0.0
	2007	38	15.8	9	0.0
	2012	61	33.0	0	0.0
6 Muranzel	2003	21	0.0	19	0.0
	2004	11	0.0	9	0.0
	2007	20	5.0	8	0.0
	2012	41	2.0	0	0.0
7 Torreira	2003	26	0.0	19	0.0
	2004	41	17.1	6	0.0
	2007	42	4.8	3	0.0
	2012	43	7.0	0	0.0
8 Ponte Varela	2003	15	0.0	25	0.0
	2004	20	0.0	20	0.0
	2007	35	2.9	8	0.0
	2012	53	34.0	5	0.0
9 Marina de Ovar	2003	29	0.0	11	0.0
	2004	0	0.0	0	0.0
	2007	10	80.0	1	0.0
	2012	27	78.0	0	0.0
10 Murtosa	2003	20	0.0	20	0.0
	2004	37	27.0	14	0.0
	2007	0	0.0	0	0.0
	2012	0	0.0	0	0.0

2.2. Effect of animal size, parasitism and TBT sediment contamination on male penis length

An OLS regression was performed to analyse the effect of SH, parasitism and TBT sediment contamination on male penis length (MPL) for data concerning the 2012 survey. The results reveal a significant and positive effect of parasitism on the levels of MPL, but curiously TBT has a highly significant negative effect on this parameter. On the contrary, SH has no significant effect on male penis length (Table 10).

Table 10 - Linear least squares regression results for the variable male penis length (MPL). β_0, β_1, β_2; β_3: coefficients for each parameter; SH: shell height; Par: male with or without parasites; TBT: concentration of TBT in sediments; ε: associated error. Adjusted $R^2 = 0.28$. F-statistic = 13.3 (92 degrees of freedom; $P = 2.8 \times 10^{-7}$). N=96

$MPL = \beta_0 + \beta_1 SH + \beta_2 Par + \beta_3 TBT + \varepsilon$

Coefficients	Value	*t*-value	*p*-value	
Intercept	2.239	4.522	1.8×10^{-5}	***
SH	0.126	1.692	9.4×10^{-2}	n.s.
Par	0.442	3.012	3.4×10^{-3}	**
TBT	-0.603	-5.781	1.0×10^{-7}	***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s.: not significant.

3. Temporal evolution of *H. ulvae imposex* in Ria de Aveiro

3.1. Seasonal evolution

The monthly variation of *imposex* levels and female gametogenesis stages in *Hydrobia ulvae*, between December of 1999 and December of 2000, is shown in Figure 12. From December 1999 to February 2000 there is a progressive increase in the number of females with stage V (partially spent), denoting a rise in the spawning activity along winter. Thereafter we assisted to a decrease of spawning and an increase of females in early (stage II) and late recovery (stage III) that start to appear by May 2000. From May to October 2000, a gradual increase in the number of females

in late recovery is evident, revealing an intense gametogenic activity in this part of the year. Although spawning occurs along these latter months, since females at stages V (partially spent) and VI (totally spent) also appear in this period, the highest spawning activity happens again in December 2000 as the majority of females in this month present stages V and VI. Overall, the reproductive cycle shows a very tenuous seasonal pattern with a highest spawning activity during the winter and a highest recovery during the summer. Nevertheless, ripe females (stage IV) and spawned females (stage V) occurred roughly throughout the year, which denotes that the population can reproduce almost continuously (Fig. 12a).

Only a-type VDS stages were observed during the entire period of this study. All females affected by *imposex* exhibit VDS stages 1 and 2 and VDSI varied between a minimum value of 0.9 in December of 1999 and a maximum of 1.2 in July of 2000, without an evident seasonal pattern (Fig. 12b). The FPL attained the lowest value of 0.27 mm in December 1999 and a maximum of 0.50 mm in July. Although higher values were roughly observed from March to July, there is a lack of a clear seasonality in this parameter as well (Fig. 12b). The percentage of females with *imposex* (%I) fluctuated between 80% in October and 97% in July with no evident temporal trend (Fig. 12c). VDSI was significantly correlated with FPL ($r=0.67$, $P<0.05$) and %I ($r=0.80$, $P<0.05$) across months. No significant correlation was found between these parameters and the average shell height, probably because the animal size was maintained nearly constant along the year (SH between 6.56 and 6.82 mm, see Fig. 12c) as a methodological requirement to avoid interference of this factor on *imposex* levels in this specific study.

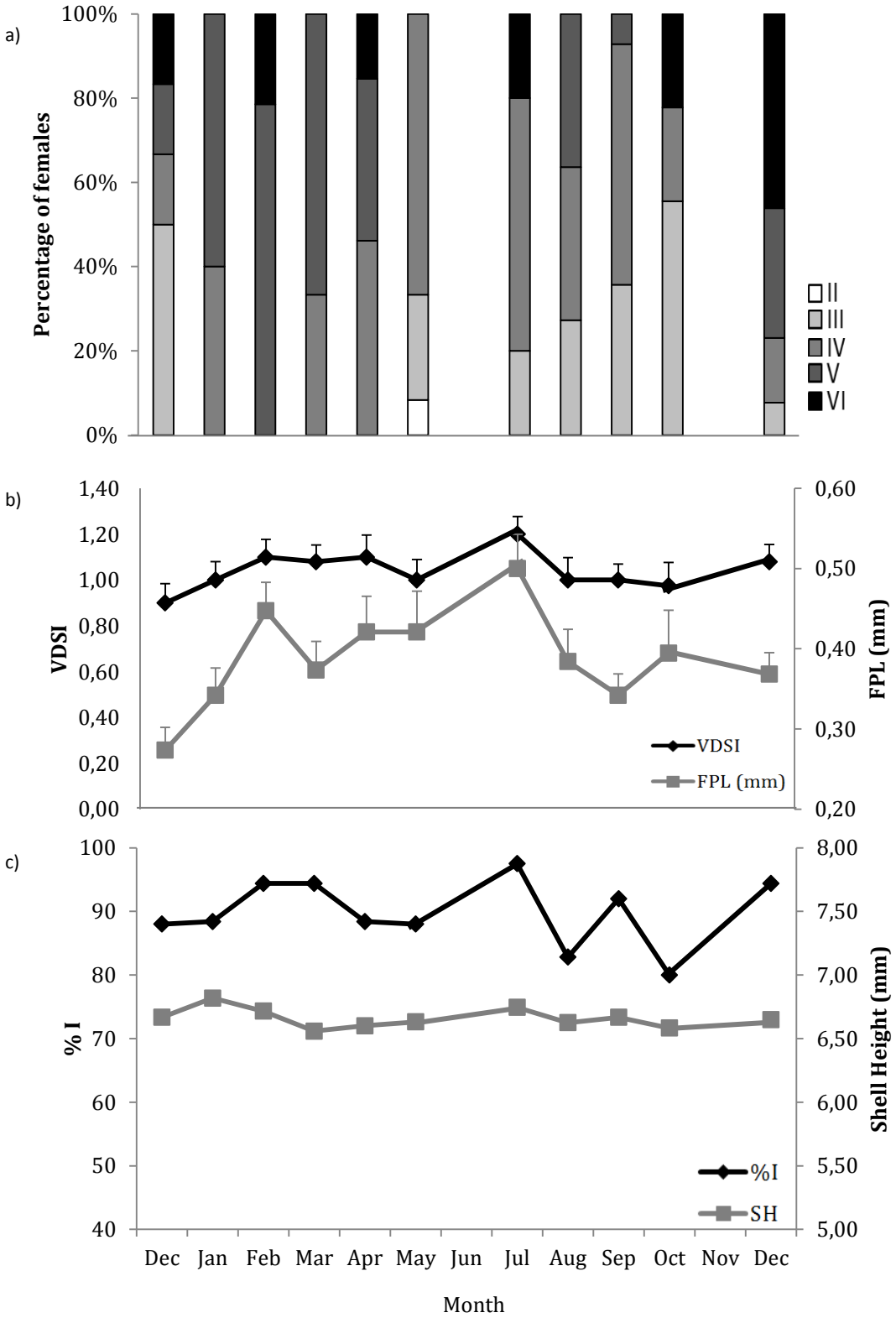

Figure 12 – *Hydrobia ulvae.* Female gametogenesis stages (a) and *imposex* parameters (b-c) in Ermida (St. 3, Ria de Aveiro) between December 1999 and December 2000. VDSI: vas deferens sequence index; FPL: female penis length; % I: percentage of *imposex* affected females.

3.2. Multi year evolution: from 1998 to 2012

Figures 13 and 14 show the evolution of *H. ulvae* VDSI and %I levels in Ria de Aveiro since the first survey performed in 1998 till the present year. In this data set parasitized females were discarded from the analysis. It is evident from the observation of this figure that there has been a slight increasing trend of *imposex* along the last 15 years. Furthermore, it is clear that in 1998 there was an heterogeneous distribution pattern of *imposex* levels in the study area that has become less pronounced as time passed. Presently, as stated in section 1.2, all sampled populations of *H. ulvae* exhibit more or less similar values of *imposex* conducing to a roughly uniform spatial distribution of this phenomenon in Ria de Aveiro. The rise of *imposex* in this species is surprising as bans on the use of TBT antifouling paints are in place since 2003 (EC Regulation 782/2003) and 2008 (IMO AFS Convention), which have certainly contributed to a slowdown of TBT inputs into Ria de Aveiro. To confirm whether this apparent increase of *imposex* is significative or not, we proceed with a regression analysis of *imposex* data concerning all non-parasitized individual females from 2003 survey onwards, taking this year as a reference because it is the starting point. The response variable is the *imposex* parameter VDS or FPL. The percentage of females affected by *imposex* is a value estimated for an entire sample and thus not subjected to this type of regression analysis. For the regressions the SH is taken into account because, as seen previously, this parameter has a significant effect on *imposex* and may present oscillations between different surveys. Besides, the sampling sites are also parameters included in the analysis due to site-specific effects they may exert on *imposex*; in this case St. 8 is considered the reference sampling site because *H. ulvae* here generally presents the lowest levels of *imposex* among all surveyed sites in Ria de Aveiro, but any other site could be alternatively selected as reference.

In what concerns to the vas deferens sequence, the results point out for a non-significant change of VDS from 2003 to 2004 but, on the contrary, a highly significant increase from 2003 to 2007 and from 2003 to 2012 (see Table 11). To check for differences among middle years we test the null hypotheses that VDS is not different

for each pair of surveys 2004/2007 (statistic=13.59, $P=2.3x10^{-4}$), 2004/2012 (statistic=46.56, $P=8.9x10^{-12}$) and 2007/2012 (statistic=9.78, $P=1.8x10^{-3}$), which is rejected in all cases, i.e., there is an increase in VDS between the above mentioned years. SH and sampling site have a significant effect on VDS (Table 11), but they act as control variables in the regression and so their effect is removed from the temporal evolution described above, turning the analysis much more robust. As for comparisons between sampling sites, the results denote that St.8 is not significantly different from Sts. 4, 6 and 10, which means that, in the overall period under study, populations at these four sites present lower VDS values comparing to other places in Ria de Aveiro.

Regarding the size of the female penis, the results show a significant increase of FPL from 2003 to 2004 and a highly significant increase from 2003 to 2012, though differences between 2003 and 2007 render non-significant (see Table 12). To check for differences between each pair of surveys 2004/2007 (F=2.48, P=0.12), 2004/2012 (F=2.23, P=0.14) and 2007/2012 ($F=10.08$, $P=1.5x10^{-3}$), the null hypothesis is rejected only in the last pair of years, i.e., there is only a significant increase in FPL between 2007 and 2012. Again, SH and sampling site have a significant effect on FPL (Table 12) and, as stated above, since they act as control variables, their effect is removed from the temporal evolution described above. Comparisons between sampling sites gave about the same results as for VDS, i.e., St.8 is not significantly different from Sts. 4, 6 and 10 and so these four sites present globally lower FPL values for the entire decade under analysis.

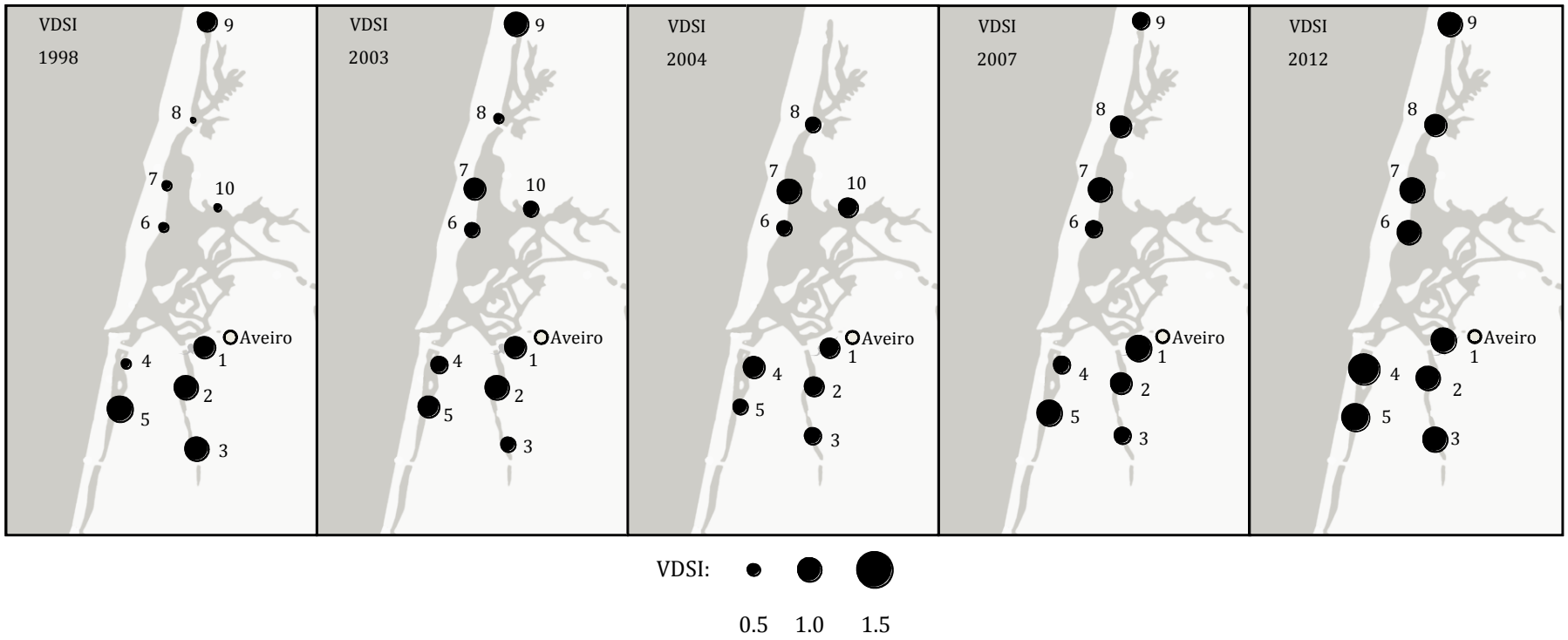

Figure 13 – *Hydrobia ulvae*. Spatio-temporal evolution of *imposex* VDSI levels in Ria de Aveiro between 1998 (Barroso et al., 2000), 2003, 2004, 2007 (Galante-Oliveira et al., 2010a) and 2012 (presente study) surveys. VDSI: vas deferens sequence.

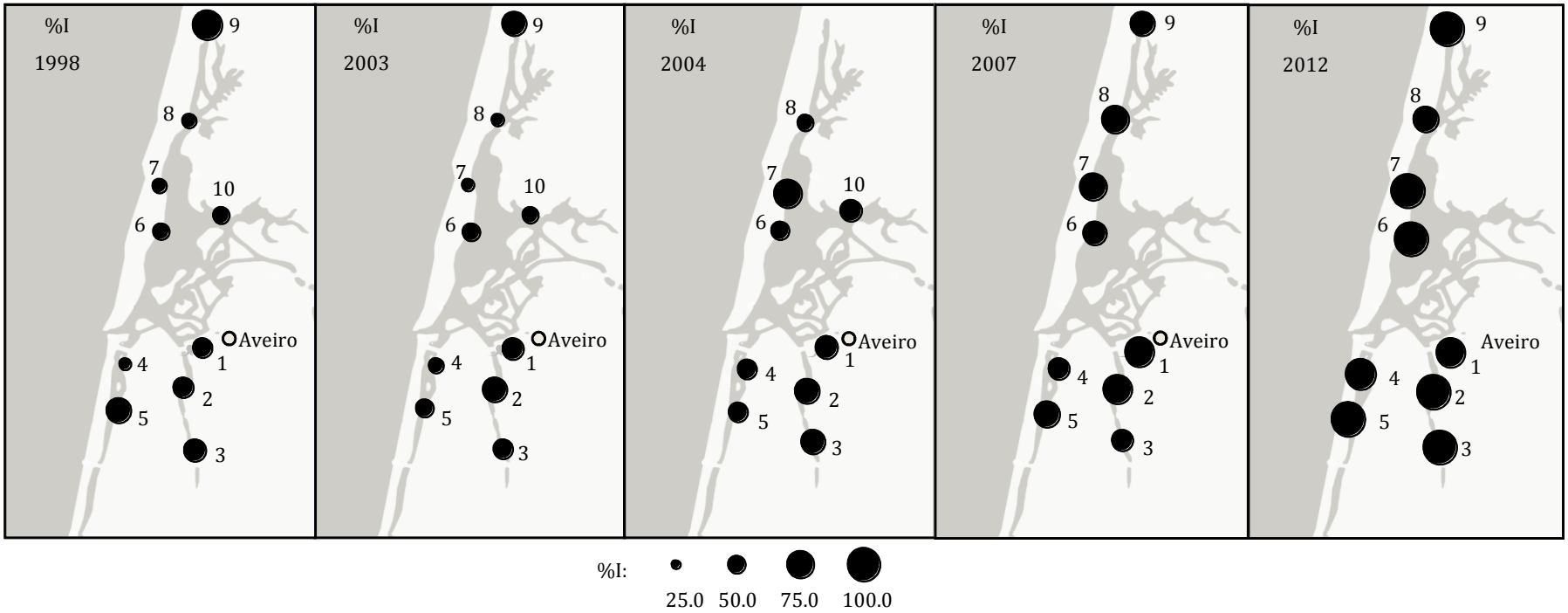

Figure 14 – *Hydrobia ulvae*. Spatio-temporal evolution % I levels in Ria de Aveiro between 1998 (Barroso et al., 2000), 2003, 2004, 2007 (Galante-Oliveira et al., 2010a) and 2012 (presente study) surveys. % I: percentage of affected females by *imposex*.

Table 11 – Results of the latent variable logit model (ordered logit model) for the ordinal variable Vas Deferens Sequence (VDS). VDS*: latent continuous variable of the ordinal VDS; β_0, β_1, β_2, … , β_{10}, β_{11}, β_{12}: coefficients for each parameter; SH: shell height; 2003, 2004, 2007 and 2012: years when *imposex* surveys were conducted; St. 1, St. 2, St. 3, … , St.8, St. 9, St. 10: sampling sites where *imposex* surveys were performed in Ria de Aveiro; ε: associated error. The year 2003 and St. 8 were used as reference year and reference sampling site for temporal and spatial comparisons, respectively. N=1427 females.

$VDS^* = \beta_0\,SH + \beta_1\,2004 + \beta_2\,2007 + \beta_3\,2012 + \beta_4\,St.\,1 + \beta_5\,St.\,2 + \beta_6\,St.\,3 + \ldots + \beta_{11}\,St.\,9 + \beta_{12}\,St.\,10 + \varepsilon$

Coefficients	Value	*t*-value	*p*-value	
Intercepts:				
0|1	1.511	3.728	1.9×10^{-4}	***
1|2	4.826	11.258	<0.001	***
2|3	7.881	14.293	<0.001	***
Parameters:				
SH	0.286	4.347	1.4×10^{-5}	***
2004	0.120	0.774	4.4×10^{-1}	n.s.
2007	0.745	4.623	3.8×10^{-6}	***
2012	1.242	7.868	3.6×10^{-15}	***
St. 1	0.822	3.776	1.6×10^{-4}	***
St. 2	0.994	3.938	8.2×10^{-5}	***
St. 3	0.430	2.042	4.1×10^{-2}	*
St. 4	0.184	0.803	4.2×10^{-1}	n.s.
St. 5	0.741	3.231	1.2×10^{-3}	**
St. 6	0.400	1.663	9.6×10^{-2}	n.s.
St. 7	0.876	3.906	9.4×10^{-5}	***
St. 9	1.217	3.471	5.2×10^{-4}	***
St. 10	0.405	1.417	1.6×10^{-1}	n.s.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s.: not significant.

Table 12 - Linear least squares regression results for the variable female penis length (FPL). β_0, β_1, β_2, β_3, … , β_{11}, β_{12}, β_{13}: coefficients for each parameter; SH: shell height; 2003, 2004, 2007 and 2012: years when *imposex* surveys were conducted; St. 1, St. 2, St. 3, … , St.8, St. 9, St. 10: sampling sites where *imposex* surveys were performed in Ria de Aveiro; ε: associated error. The year 2003 and St. 8 were used as reference year and reference sampling site for temporal and spatial comparisons, respectively. Ajusted R^2 = 0.15. F-statistic = 19.7 (1413 degrees of freedom; P<2.2 x 10-16); N=1427 females.

FPL = β_0 + β_1 SH + β_2 2004 + β_3 2007 + β_4 2012 + β_5 St. 1 + β_6 St. 2 + … + β_{12} St. 9 + β_{13} St. 10 + ε

Coefficients	Value	*t*-value	*p*-value	
Intercept	-0.382	-6.009	2.37 x 10^{-9}	***
SH	0.101	9.744	<2.0 x 10^{-16}	***
2004	0.049	2.050	4.1 x 10^{-2}	*
2007	0.007	0.294	7.7 x 10^{-1}	n.s.
2012	0.088	3.628	3.0 x 10^{-4}	***
St. 1	0.154	4.485	7.9 x 10^{-6}	***
St. 2	0.404	10.189	<2.0 x 10^{-16}	***
St. 3	0.752	2.190	2.9 x 10^{-2}	*
St. 4	0.052	1.442	1.5 x 10^{-1}	n.s.
St. 5	0.102	2.859	4.3 x 10^{-3}	**
St. 6	0.069	1.766	7.8 x 10^{-2}	n.s.
St. 7	0.111	3.117	1.9 x 10^{-3}	**
St. 9	0.175	3.244	1.2 x 10^{-3}	**
St. 10	0.085	1.890	5.9 x 10^{-2}	n.s.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s.: not significant.

IV. Discussion

The main objective of the current work is to assess the temporal evolution of *imposex* levels of *H. ulvae* in Ria de Aveiro since the first survey performed in this area (1998) till the present days, i.e., for a period of 15 years. The current study is integrated in a more extensive program aiming to monitor the evolution of TBT pollution in Ria de Aveiro, for which chemical and biological monitoring has been conducted throughout the entire area for the above mentioned period. Of particular interest is the evaluation of the impact of the TBT antifouling bans that entered into force in 2003 (EC Regulation 782/2003) and 2008 (IMO AFS Convention), which would contribute to a slowdown of TBT inputs into this estuarine system and to a lessening of the adverse biological effects caused by this harmful toxicant. The global results of this vast monitoring program indicate a very clear decline of TBT concentration in water (Barroso et al., 2004; Sousa et al., 2007; Sousa et al., 2009b), sediments and biota (mollusc bivalves and gastropods; Barroso et al., 2002; Barroso et al., 2005; Barroso et al., 2011; Galante-Oliveira et al., 2006; Galante-Oliveira et al., 2009; Galante-Oliveira et al., 2011; Rato et al., 2006; Rato et al., 2009a; Rato et al., 2009b; Sousa et al., 2005; Sousa et al., 2009a) since 2003, accompanied by a reduction of *imposex* in key bioindicators such as *Nassarius reticulatus* (Barroso et al., 2002; Barroso et al., 2005; Barroso et al., 2011; Laranjeiro et al., 2010; Rato et al., 2006; Rato et al., 2009a; Rato et al., 2009b; Sousa et al., 2005; Sousa et al., 2009a) and *Nucella lapillus* (Barroso et al., 2002; Galante-Oliveira et al., 2006; Galante-Oliveira et al., 2009; Galante-Oliveira et al., 2011; Laranjeiro et al., 2010) inside and outside Ria de Aveiro, as well as a decrease of the shell thickness index in *Crassostrea gigas* at the single site where this species is produced – an aquaculture facility located near St. 4 in Mira Channel (Galante-Oliveira et al., 2009). Despite all evidences pointing for a reduction of TBT inputs into Ria de Aveiro since 2003 and a consequent recovery of the ecosystem, surprisingly the mud snail *H. ulvae* shows a global rise of *imposex* values since that date. In fact, as shown in previous chapters, the percentage of females affected by *imposex* increased progressively in the entire area and the VDS and FPL also rose significantly after the implementation of the EC Regulation 782/2003. These contradictory trends are difficult to explain but the following

paragraphs will examine and hypothesise the possible reasons that are in the base of this apparent incongruity and may clarify why the mud snail acts as a counter current bioindicator.

Before we proceed with a more detailed analysis, it is important to exclude any methodological flaws that could lead to biased results when using the mud snail as an *imposex* indicator. There are four main concerns regarding this issue: (i) extreme seasonal fluctuations of *imposex* levels at fixed sites; (ii) strong variation of the size of the bioindicator specimens between different surveys; (iii) bioindicator longevity that is lower than the frequency of biomonitoring; (iv) considerable mobility of bioindicator specimens between locations. The first point (i) was checked in the current study using St.3 as a case study. Indeed, it was not found a pronounced variation of VDSI, FPL and %I in the mud snail at this site along one entire year. Moreover, the sampling surveys that have been performed in Ria de Aveiro since 1998 always occur between February and July, thus reducing even more any seasonality interference on the results. It should be mentioned that male penis size in prosobranchs may typically vary throughout the year with the reproductive cycle, which may affect RPL, but this index was not used for temporal comparisons in the current work (it is provided in Table 5 for the sake of completeness reader information). The second point (ii) is important as it is demonstrated in the current study that SH has a significant effect on *imposex* levels; however, this parameter is taken into account as a control variable in the statistical regressions and so the variability of female size between surveys is removed from the analysis. The third point (iii) is particularly relevant when pollution is declining since *imposex* is largely irreversible and a reduction can only be well detected when older (most affected females) of the indicator species are substituted by younger ones. This is easily accomplished if the longevity of the bioindicator is lower that the frequency of monitoring, as it is clearly the case in the present study: *H. ulvae* presents a longevity of just 2 years in Ria de Aveiro (Silva, 2002) which is below the frequency of monitoring at least for 1998/2003, 2003/2007 and 2007/2012 and between these years. Finally, the fourth point (iv) is of considerable interest as it is known that *H. ulvae* juvenile (SH <2 mm) dispersal by floating occurs in Ria de Aveiro, which may

cause mixing of mud snails from different TBT polluted sites. However, this dispersion behaviour at Ria de Aveiro is restricted to a very short period in the youngest stage. Besides, we minimize this problem by using adults in the monitoring programmes and by assessing the evolution of *imposex* in the whole study area instead of isolated sites.

After checking that the methodology used in the current work is quite robust, we return to the fundamental basis of the current discussion: if the temporal evolution of *imposex* in the mud snail goes in an opposite direction than that of TBT pollution, it is questionable if *H. ulvae* is an adequate indicator in the study area. Galante-Oliveira et al. (2010a) suggest that the mud snail may show a slower declining rate *imposex* when comparing to other prosobranch bioindicators because this species ingests sediments and by this way a higher and longer intake of TBT may occur because this compound tends to persist in sediments for many years. This could explain why *Nassarius reticulatus* and *Nucella lapillus* presented a very fast *imposex* recovery in Ria de Aveiro since 2003 in contrast with the mud snail. Still, the mud snail did not reflect at all the decline of environmental tributyltin levels that has occurred in the sediments of Ria de Aveiro since 2003. A good example of this is the evolution of the levels of TBT contamination in sediments: in 1998 at Sts. 1, 3, 4, 5, 7, 8 and 9 were 65, 19, 19, 84, 30, 8 and 10 ng TBT-Sn. g^{-1} dw, respectively (Barroso et al., 2000), whilst in 2012 these values declined to 5.2, 11, 3.1, 29, 1.5, 3.2 and 2.8 ng TBT-Sn. g^{-1} dw, for the same ordered sites. This sharp decrease in TBT sediment contamination clearly contradicts the increase of *imposex* observed in the mud snail for the same locations and so it seems that the diet of *H. ulvae* cannot justify per itself this discrepancy.

Other relevant fact is that levels of sediment contamination observed in 2012 are, in general, very low. This confirms the amelioration of pollution levels after the implementation of the bans on TBT antifouling paints. Although BDI revealed some new inputs of TBT, these are certainly minor ones derived from old painted vessels or from TBT remobilisation from sediments. Most importantly, TBT concentration in sediments are less than the threshold of 20 ng TBT-Sn. g^{-1} dw for which *imposex* is initiated in the mud snail (Schulte-Oehlmann et al., 1998), the single exception being

St. 5 with a value of 29 ng TBT-Sn. g^{-1} dw. This probably explains why we observe a non-significant effect of TBT sediment concentration on the mud snail *imposex* levels in the 2012 survey (Table 7 and 8) or did not find significant correlation between TBT and *imposex* parameters across sites. This also explains why there is no spatial heterogeneity in the intensity of *imposex* in the study area in 2012, i.e., there is no correlation with the proximity of ports, marinas or dockyards as seen in 1998. Moreover, the correlation between TBT concentration in sediments, in 1998 and in 2012, resulted non-significant ($r=0.41$; $P>0.05$), meaning different TBT pollution sources. Hence, it appears that the actual masculinisation of mud snail females at Ria de Aveiro is caused by a different factor than TBT.

It should be mentioned that Schulte-Oehlmann et al. (1997) cite very old reports from other researchers who had found masculinized females of *H. ulvae* exhibiting small penises well before TBT pollution existed, a phenomenon that they linked to parasitism. Schulte-Oehlmann et al. (1997) also perceived, in their study, a slight increase of parasitism in *imposex* affected females in comparison with unaffected ones and they admit that virilisation can be caused by parasitism with larval trematodes, though not being the only or decisive reason for *imposex* development, which would be driven by TBT contamination. For this reason, the authors recommend that parasitized specimens should be excluded from TBT biomonitoring programmes. The major question to be raised is if one can effectively remove all parasitized mud snails from *imposex* studies just relying on the external observation of the animals using a stereo-microscope, as it is usually done. Perhaps the complete removal of parasitized animals requires more detailed techniques such as histology, especially when initial stages of parasitism are present.

Our data corroborate the positive effect of parasitism on *imposex* levels: not only this rendered significant in the regression analysis (Tables 7 and 8) but we also found the very odd coincidence of parasitism been exclusively observed in females affected with *imposex* (Table 9). We may hypothesise that the increase of mud snail *imposex* levels in Ria de Aveiro could be related to an eventual increase of parasitism. If we were unable to discard from the analysis females exhibiting initial and undetectable stages of parasitism, this could lead to an increase of *imposex* in our

study. However, there are a few drawbacks of this deduction: firstly, there is lack of evidence for a rise of the prevalence of parasitism in the mud snails during the last years in the study area; secondly, the *imposex* levels observed in 2012 are very similar across sampling sites, despite the huge discrepancies in the percentage of parasitized females registered in those sites (varying from 0 to 78% - Table 9); thirdly, to our knowledge there is no categorical study that has experimentally proved the induction of female mud snail masculinisation by digenean parasites. Hence, more studies are needed to clarify these aspects.

In conclusion, the present study points out the fact that bioindicators have different properties and sensitivities that require an exhaustive validation regarding their proper use in environmental monitoring. In particular, *H. ulvae* has proved to be a species in which *imposex* levels have evolved in a very different way when comparing to other bioindicators like *Nassarius reticulatus* and *Nucella lapillus* over the last years in the Ria de Aveiro. The current work suggests that parasitism may be the key factor that explains this disparity, but this and other hypotheses should be further investigated in the near future.

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